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Efficacy and Safety of Leptin-Replacement Therapy and Possible Mechanisms of Leptin Actions in Patients with Generalized Lipodystrophy

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Background: Lack of leptin is implicated in insulin resistance and other metabolic abnormalities in generalized lipodystrophy; however, the efficacy, safety, and underlying mechanisms of leptin-replacement therapy in patients with generalized lipodystrophy remain unclear.

Methods: Seven Japanese patients with generalized lipodystrophy, two acquired and five congenital type, were treated with the physiological replacement dose of recombinant leptin during an initial 4-month hospitalization followed by outpatient follow-up for up to 36 months.

Results: The leptin-replacement therapy with the twice-daily injection dramatically improved fasting glucose (mean ± SE, 172 ± 20 to 120 ± 12 mg/dl, $P < 0.05$) and triglyceride levels (mean ± SE, 700 ± 272 to 260 ± 98 mg/dl, $P < 0.05$) within 1 wk. The leptin-replacement therapy reduced insulin resistance evaluated by euglycemic clamp method and augmented insulin secretion at glucose tolerance test with different responses between acquired and congenital types. Improvement of the fatty liver was also observed. The efficacy and safety of the once-daily injection were comparable to those of the twice-daily injection. The leptin-replacement therapy ameliorated macro- and microalbuminuria and showed no deterioration of neuropathy and retinopathy of these patients. The leptin-replacement therapy is beneficial to diabetic complications and lipodystrophic ones. Two patients developed antileptin antibodies but not neutralizing antibodies. The therapy was well tolerated, and its effects were maintained for up to 36 months without any notable adverse effects such as hypoglycemia, high blood pressure, or reduction of bone mineral density.

Conclusions: The present study demonstrates the efficacy and safety of the long-term leptin-replacement therapy and possible mechanisms of leptin actions in patients with generalized lipodystrophy. (***J Clin Endocrinol Metab* 92: 532–541, 2007**)

LEPTIN PLAYS A MAJOR role in the regulation of energy homeostasis (1). The plasma leptin concentration increases in proportion to the degree of adiposity (2–6). Besides the antiobesity actions, leptin has a wide range of actions including antidiabetic actions (6–8).

Generalized lipodystrophy is a heterogeneous group of diseases characterized by a profound deficiency of adipose tissue (9) and is commonly associated with severe insulin-resistant diabetes, hypertriglyceridemia, and fatty liver (10, 11). In lipoatrophic patients, these metabolic abnormalities develop as a consequence of decreased mass of the adipose tissue (12–14), and consequently, plasma leptin concentrations are markedly reduced (15). We and others demonstrated that the leptin administration or transgenic overexpression of leptin reverses the metabolic abnormalities in different mouse models of lipodystrophy, indicating that the metabolic abnormalities in lipoatrophic patients are caused mainly by a shortage of leptin (16, 17). Recently, the 4-month leptin-replacement therapy with twice-daily injection protocol was reported to improve glucose and lipid metabolism in nine female patients with lipodystrophy in the United States (18).

In the present study, we evaluated the efficacy and safety of long-term leptin-replacement therapy on seven Japanese patients with generalized lipodystrophy.

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Abbreviations: AGL, Acquired generalized lipodystrophy; CGL, congenital generalized lipodystrophy; CT, computed tomography; HbA1c, glycosylated hemoglobin; L/S, liver to spleen; r-metHuLeptin, recombinant methionyl human leptin.

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Subjects and Methods

Subjects

Eligible criteria were according to the study protocol of the National Institutes of Health (18). We evaluated seven patients with generalized lipodystrophy including two patients with acquired generalized lipodystrophy (AGL) and five patients with congenital generalized lipodystrophy (CGL). Patients with CGL were further analyzed for mutations in either *seipin* (19) or 1-acylglycerol-3-phosphate *O*-acyltransferase2 (*AGPAT2*) genes (20). Table 1 summarizes the baseline clinical characteristics of seven patients treated in the present study.

Study design

The study protocol was approved by the ethical committee of Kyoto University Graduate School of Medicine (approval number 331). Informed written consent was obtained from all subjects and their families. Recombinant methionyl human leptin (r-metHuLeptin) was provided by Amgen, Inc. (Thousand Oaks, CA). For the first year, r-metHuLeptin

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TABLE 1. Characteristics of the patients at baseline

Patient no.	1	2	3	4	5	6	7	Mean ± SE
Age (yr)	11	29	19	16	15	23	33	21 ± 3
Sex	F	M	M	F	F	F	F	
Type of lipodystrophy	AGL	CGL[a]	CGL[b]	AGL	CGL[b]	CGL[b]	CGL[a]	
Body weight (kg)	31.0	32.0	52.8	34.6	43.5	52.0	53.9	42.8 ± 3.9
BMI (kg/m^2)	16.0	14.2	19.7	13.9	17.9	21.2	20.3	17.6 ± 1.1
Body fat (%)[c]	5.0	4.7	5.0	5.8	5.4	5.7	10.0	5.9 ± 0.7
Age of DM onset (yr)	9	11	10	15	6	5	14	10 ± 1
Duration of DM (yr)	2	18	9	1	9	18	19	11 ± 3
Leptin (ng/ml)	0.92	0.82	1.23	1.15	0.90	1.23	1.40	1.09 ± 0.08
Fasting glucose (mg/dl)	208	142	105	138	247	221	130	172 ± 21
HbA1c (%)	10.0	10.3	8.8	7.9	7.7	10.2	10.2	9.3 ± 0.4
Triglyceride (mg/dl)	1941	69	1031	1246	89	254	232	695 ± 273
Total cholesterol (mg/dl)	250	183	194	298	185	285	233	233 ± 18
L/S ratio	0.78	1.23	0.95	0.35	1.12	0.88	0.73	0.86 ± 0.11
Urinary albumin (mg/d)	48.4	6.9	31.0	778	20.3	359	11.3	179.3 ± 110.5
Blood pressure (mm Hg)	114/48	120/82	126/54	92/52	108/56	104/64	108/70	110 ± 4/61 ± 5
Bone mineral density (g/cm^2)[c]	0.94	0.88	1.32	0.90	1.06	1.37	1.33	1.11 ± 0.08
Diet therapy (kcal/d)	1500	1500	1800	1500	1500	1600	1500	
Antidiabetic therapy	Pioglitazone (15 mg/d)	Glibenclamide (2.5 mg/d), voglibosie (0.6 mg/d)	Insulin (60 IU/d)	Pioglitazone (30 mg/d)	None	Pioglitazone (45 mg/d)	Insulin (20 U/d)	
Lipid-lowering therapy	None	None	None	Pravastatin (40 mg/d), bezafibrate (400 mg/d)	None	None	None	

BMI, Body mass index; DM, diabetes mellitus; L/S ratio, ratio of liver to spleen for CT attenuation values.

[a] CGL due to neither *seipin* nor *AGPAT2* mutation.

[b] CGL due to *seipin* mutation.

[c] Body fat and bone mineral density were measured by dual energy x-ray absorptiometry.

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was administered as twice-daily sc injection (18). The physiological replacement dose was estimated to be 0.02 mg/kg·d for men, 0.03 mg/kg·d for girls under 18 yr of age, and 0.04 mg/kg·d for women on the basis of information provided by Amgen. Patient 1 was treated with 100% of the replacement dose for the entire period. Patient 2 was treated with 100% for the first and second month and 200% thereafter. Patients 3–7 were treated with 50% for the first month, 100% for the second month, and 200% thereafter. All patients were evaluated as inpatients for the first 4 months. After discharge, patients attended local clinics for every leptin injection, and all of the leptin injections were done by medical doctors because self-injection of r-metHuLeptin, which was not approved as a drug, was not permitted in Japan. Each patient had been prescribed a diet of fixed calories indicated in Table 1 beginning at least 2 months before the initiation of leptin-replacement therapy, and this was not altered throughout the therapy. The dose of antidiabetic and lipid-lowering drugs was tapered or the treatment discontinued as needed. After 12 months of twice-daily leptin treatment, we reduced the dosing frequency to once daily without change of total daily dose. At present, total duration of leptin-replacement therapy was 36 months for patient 1 and 2, 24 months for patient 3, 18 months for patient 4, 8 months for patient 5, and 2 months for patients 6 and 7.

Biochemical analysis

Plasma leptin levels were determined by the immunoassay (Linco, St. Charles, MO). Plasma glucose, serum triglycerides, total cholesterol, alanine aminotransferase, aspartate aminotransferase, and serum and urine creatinine levels were determined according to standard methods with the use of automated equipment. Glycosylated hemoglobin (HbA1c) levels were measured by ion-exchange HPLC. Serum insulin levels were determined by immunoassays (Shibayagi Co., Ltd., Gunma, Japan). Urine albumin excretion was assayed with a human albumin ELISA kit (Sanko Junyaku Co., Ltd., Tokyo, Japan). Antibodies to leptin in serum was tested with the use of a solid-phase RIA, and the potential neutralizing effects of antibodies on leptin bioactivity were assessed in an *in vitro* bioassay developed by Amgen (Thousand Oaks, CA) (21).

Procedures

Body fat and whole-body bone mineral density were determined by dual-energy x-ray absorptiometry (QDR-2000; Hologic Inc., Bedford, MA). The oral glucose tolerance test (75 g) was performed after an overnight fast. In patients under insulin therapy, insulin injection was stopped from the previous night. The Σ values of plasma glucose (PG) levels and serum insulin (IRI) levels were calculated by the sum of the values at 0, 30, 60, 90, 120, and 180 min after administration. Insulin action on glucose uptake in peripheral tissues was evaluated using the hyperinsulinemic-euglycemic glucose clamp technique (22). Fatty liver was diagnosed by both ultrasound and computed tomography (CT) imaging. Liver volume was calculated with the use of CT imaging. Lipid contents of liver and skeletal muscle were determined by magnetic resonance imaging performed on a 1.5-T system (Magnetom Symphony; Siemens Medical System, Erlangen, Germany). The signal intensity of the same region on both the in-phase image (I_{in}) and the out-of-phase image (I_{out}) was measured. The fat index (FI) was defined by the following formulae: $FI = (I_{in} - I_{out}) / I_{in}$. Tissue lipid content was calculated using FI as previously reported (23).

Statistical analysis

Data were expressed as the mean ± SE. Comparison between baseline data and data obtained at various times was assessed by ANOVA and completed by Fisher's probable least-significant difference test, as required.

Results

Baseline characteristics

Three of five CGL patients were homozygous for the same nonsense mutation (R275X) of the *seipin* gene as we previously reported (Table 1) (24). The remaining CGL patients had neither *seipin* nor *AGPAT2* gene mutation (24, 25). All the patients had markedly decreased body fat, hypoleptinemia, and uncontrolled diabetes with high fasting glucose levels and HbA1c levels, despite the diet and exercise therapy and the use of oral antidiabetic drugs or insulin. Their age of onset and duration of diabetes are also summarized in Table 1. Three of seven patients had marked fasting hypertriglyceridemia at the level above 1000 mg/dl. The mean ± SE of the total cholesterol level was 233 ± 18 mg/dl. Five patients were diagnosed to have fatty liver, and their ratios of liver to spleen (L/S ratio) for CT attenuation values were under 0.95. Four of seven patients had elevated urine albumin excretion (>30 mg/d), and two of them had macroalbuminuria (>300 mg/d). All the patients showed normal blood pressure (mean ± SE, 110 ± 4/61 ± 5 mm Hg) and bone mineral density (mean ± SE, 1.11 ± 0.08 g/cm^2).

High compliance of leptin-replacement therapy

All of the leptin injections were done by medical doctors. For the initial 4 months, all the patients received 100% of scheduled leptin injections as inpatients. After discharge, patients attended local clinics for every leptin injection and received over 98% as outpatients thereafter.

Achievement of physiological replacement of leptin

At any dose, peak plasma levels occurred 2 h after the leptin injection. The peak plasma leptin levels at the doses of 50, 100, and 200% under the protocol of twice-daily injections were 4.05 ± 0.19, 9.80 ± 1.70, 18.95 ± 1.58 (mean ± SE) ng/ml, respectively. The peak plasma leptin level of the 400% dose under the protocol of once-daily injections was 34.48 ± 2.11 (mean ± SE) ng/ml. Thus, the elevations of plasma leptin level were dose dependent, and physiological replacement was achieved as expected.

Rapid effects on glucose and triglyceride levels

The fasting plasma glucose levels decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 172 ± 20 mg/dl at baseline *vs.* 120 ± 12 mg/dl after 7 d, $P < 0.05$) (Table 2). By 4 months, all the patients, except patient 6, were able to discontinue all of the antidiabetic drugs (Table 1). Patient 6 could reduce the dose of the antidiabetic drug by 2 months.

The fasting triglyceride levels also decreased day by day in all the patients, and a significant reduction was achieved within 7 d (mean ± SE, 700 ± 272 mg/dl at baseline *vs.* 260 ± 98 mg/dl after 7 d, $P < 0.05$) (Table 2). Lipid-lowering drugs of patient 4 were able to be discontinued by 4 months.

Glucose tolerance tests

As shown in Fig. 1A, the mean plasma glucose levels in response to the oral 75-g glucose load were dramatically improved already at 1 month and were maintained at 2 and 4 months in all patients. The insulin levels were distinctly low before the treatment in both AGL and CGL patients (Fig. 1, B and C). The changes after the initiation of the leptin-replacement therapy of serum insulin levels showed a marked contrast between AGL and CGL patients. Glucose-induced insulin secretion was dramatically improved already at 1

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TABLE 2. Changes of fasting plasma glucose and serum triglyceride levels for first month of the leptin-replacement therapy

Patient no.	Fasting plasma glucose (mg/dl)					Fasting serum triglyceride (mg/dl)				
	Baseline	1 d	3 d	7 d	30 d	Baseline	7 d	14 d	21 d	28 d
1	208	177	160	108	76	1941	653	210	204	218
2	142	115	119	102	94	69	51	49	72	50
3	105	100	89	105	108	1031	122	108	97	115
4	138	125	126	96	108	1246	589	320	425	496
5	247	227	204	182	151	254	399	134	151	194
6	221	185	169	141	130	89	63	54	131	74
7	141	135	133	105	125	269	54	85	95	99
Mean ± SE	172 ± 20	152 ± 17	143 ± 14	120 ± 12[a]	113 ± 9[b]	700 ± 272	260 ± 98[a]	110 ± 38[b]	168 ± 46[b]	178 ± 58[b]

[a] $P < 0.05$ compared to baseline.
[b] $P < 0.01$ compared to baseline.

month in AGL patients (Fig. 1B), whereas no apparent improvement in insulin secretion was observed even after 4 months of the therapy in CGL patients (Fig. 1C). To evaluate the ability of insulin secretion, we calculated the values of ΣIRI/ΣPG in a 75-g oral glucose tolerance test. The values of ΣIRI/ΣPG were substantially increased at 1 month in two AGL patients, and additional increases were observed at 2 and 4 months, whereas those in five CGL patients remained unchanged even after 4 months of the therapy (Fig. 1D).

Hyperinsulinemic-euglycemic clamp study

The glucose infusion rates during the hyperinsulinemic-euglycemic clamp study were distinctly low at baseline in all the patients (mean ± SE, 2.5 ± 0.3 mg/kg·min; range, 1.60–3.6 mg/kg·min). The increase of glucose infusion rate was observed but not statistically significant at 1 month on the treatment (mean ± SE, 3.7 ± 0.3 mg/kg·min, $P = 0.062$ *vs.* at baseline). A significant increase was achieved at 2 months (mean ± SE, 4.4 ± 0.4 mg/kg·min, $P < 0.01$ *vs.* at baseline) and an additional increase was observed at 4 months (mean ± SE, 5.6 ± 1.0 mg/kg·min, $P < 0.001$ *vs.* at baseline). By contrast to insulin secretion, no apparent difference between AGL and CGL patients was observed on the changes of insulin sensitivity.

In patient 4, the hyperinsulinemic-euglycemic clamp study was performed at 10 d. A substantial increase of glucose infusion rate was detected already at 10 d (2.52 mg/kg·min at baseline and 4.63 mg/kg·min at 10 d) and again at 1 month (4.59 mg/kg·min), which was comparable to that at 4 months (5.06 mg/kg·min).

Effects on fatty liver

Five of seven patients were diagnosed to have apparent fatty liver. The L/S ratios of CT attenuation value in five of seven patients were 0.74 ± 0.10 (mean ± SE) (Table 1). The L/S ratio of CT attenuation value in these patients improved from 0.74 ± 0.10 (mean ± SE) to 1.09 ± 0.06 (mean ± SE) by 2 months and further improved thereafter. Consistent with this, in these patients, the alanine aminotransferase level decreased from 80.5 ± 24.2 to 32.3 ± 4.6 U/liter (mean ± SE), and the ASL level decreased from 42.3 ± 11.1 to 21.5 ± 4.3 U/liter (mean ± SE) by 2 months, and these values were also further improved thereafter. The liver volume also decreased in all patients who had fatty liver at baseline (mean ± SE,

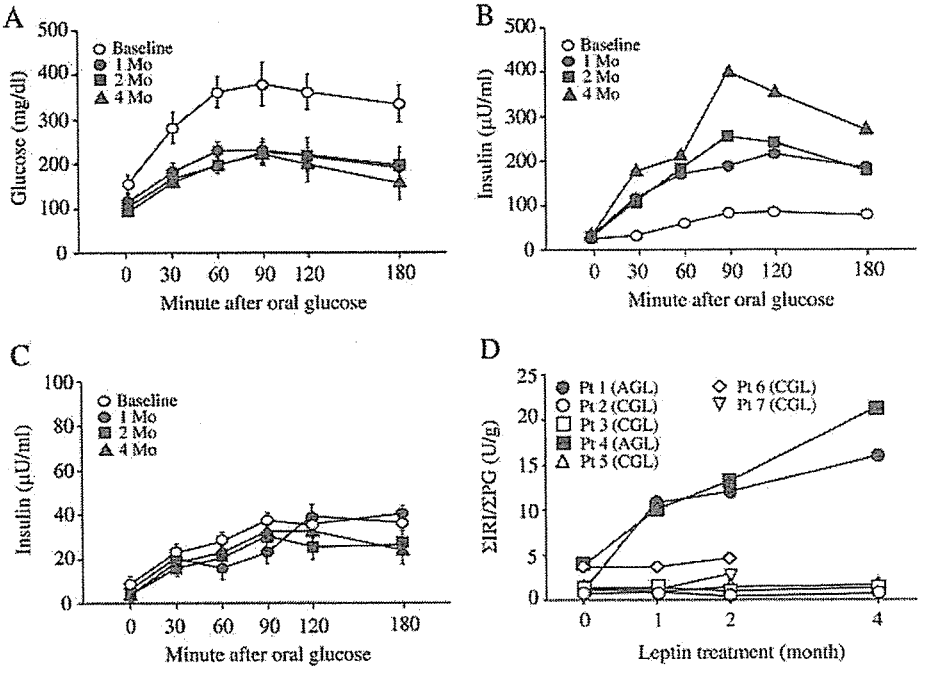

FIG. 1. Mean ± SE plasma glucose levels in all seven patients (A), mean serum insulin levels in two AGL patients (B), mean ± SE serum insulin levels in five CGL patients (C), and ΣIRI/ΣPG of each patient (D) after an oral 75-g glucose-tolerance test before and after 1, 2, and 4 months of the leptin-replacement therapy. ΣIRI, Sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣPG, sum of the plasma insulin levels before and 30, 60, 90, 120, and 180 min after an oral 75-g glucose load; ΣIRI/ΣPG, value of ΣIRI divided by ΣPG.

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1.88 ± 0.12 l at baseline to 1.50 ± 0.10 l at the end of the second month).

In patient 4, measurements of tissue lipid content were performed using magnetic resonance imaging before and after 3 and 10 d and 1, 2, and 4 months of the leptin-replacement therapy. At baseline, lipid content in her liver was clearly increased (29.0%), whereas that in her skeletal muscle was not increased (4.3%). After the leptin-replacement therapy, a distinct change of lipid content in the liver was not detected at 3 and 10 d (31.5 and 28.4%, respectively), but a substantial and gradual decrease was detected at 1 month and again at 2 and 4 months (23.5, 17.5, and 9.6%, respectively). On the other hand, in the skeletal muscle, no distinct change of lipid content was detected even at 4 months (4.2%).

Metabolic controls after discharge for 8 months

After the initial 4 months of hospitalization, the patients were continuously followed as outpatients on the protocol of twice-daily injection. Their fasting glucose levels (Fig. 2A), HbA1c levels (Fig. 2B), glucose infusion rates during the hyperinsulinemic-euglycemic clamp study (Fig. 2C), triglyceride levels (Fig. 2D), total cholesterol levels (Fig. 2E), and liver volumes (Fig. 2F) at 8 and 12 months were almost unchanged when compared with those at 4 months, the end of the hospitalization.

Once-daily leptin injection

After 12 months of twice-daily leptin injection, the treatment protocol was altered to once-daily dosing without change of total daily dose in patient 1–4. The alteration of leptin injection protocol did not affect the plasma glucose levels before breakfast, lunch, and dinner in four patients (Fig. 3, A–C). Consistent with these results, HbA1c (Fig. 2B) levels and results of the 75-g oral glucose tolerance test (data not shown) in these patients did not change after the protocol alteration. Likewise, glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, triglyceride levels, total cholesterol levels, and liver volumes were unchanged after the alteration of the treatment protocol (Fig. 2, C–F).

Long-term effects

The duration of leptin-replacement therapy was 36 months for patients 1 and 2, 24 months for patient 3, and 18 months for patient 4. The fasting plasma glucose levels and HbA1c levels were well controlled throughout the therapy period (Fig. 2, A and B). The improved glucose infusion rates during the hyperinsulinemic-euglycemic clamp study, decreased triglyceride and total cholesterol levels, and liver volumes after 4 months of leptin-replacement therapy as inpatients

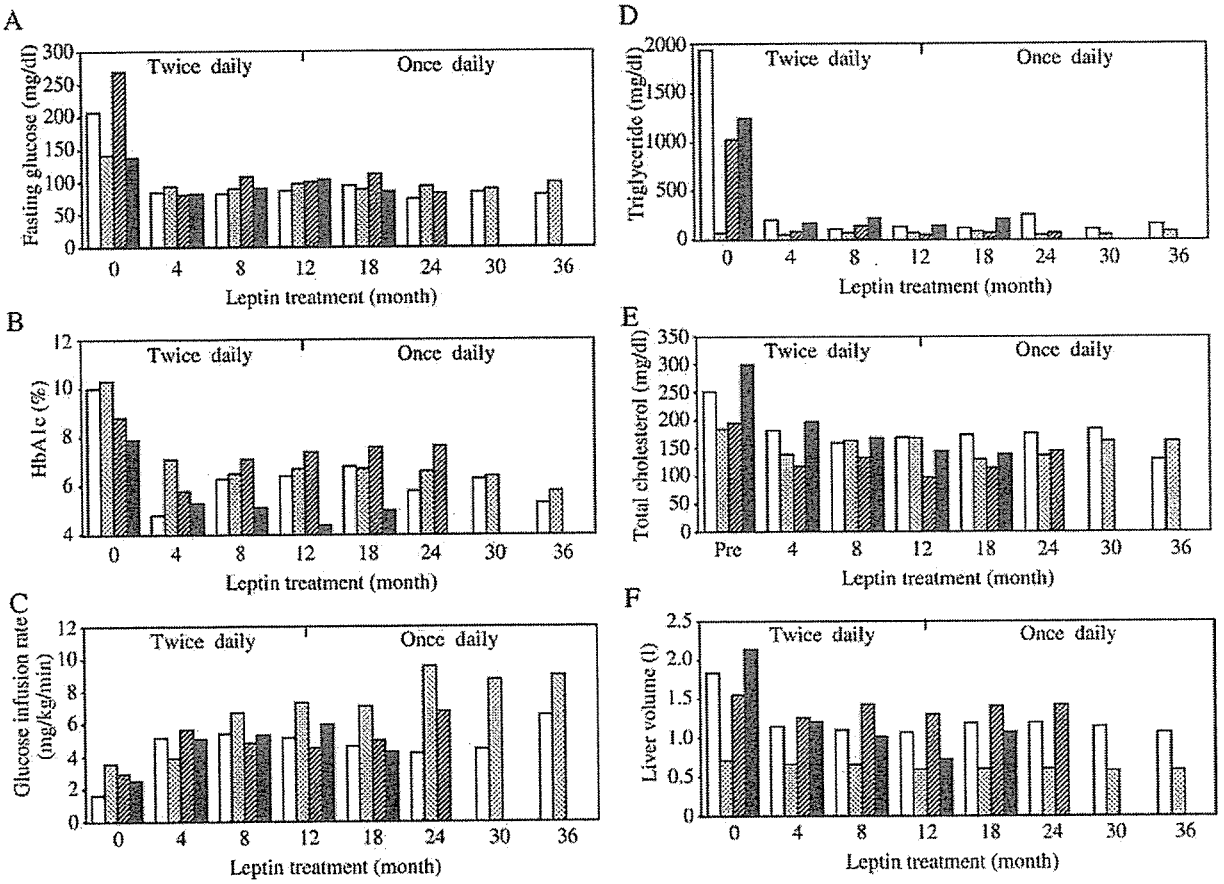

FIG. 2. Fasting plasma glucose levels (A), HbA1c levels (B), glucose infusion rates during the hyperinsulinemic euglycemic clamp study (C), triglyceride levels (D), total cholesterol levels (E), and liver volumes (F) before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*).

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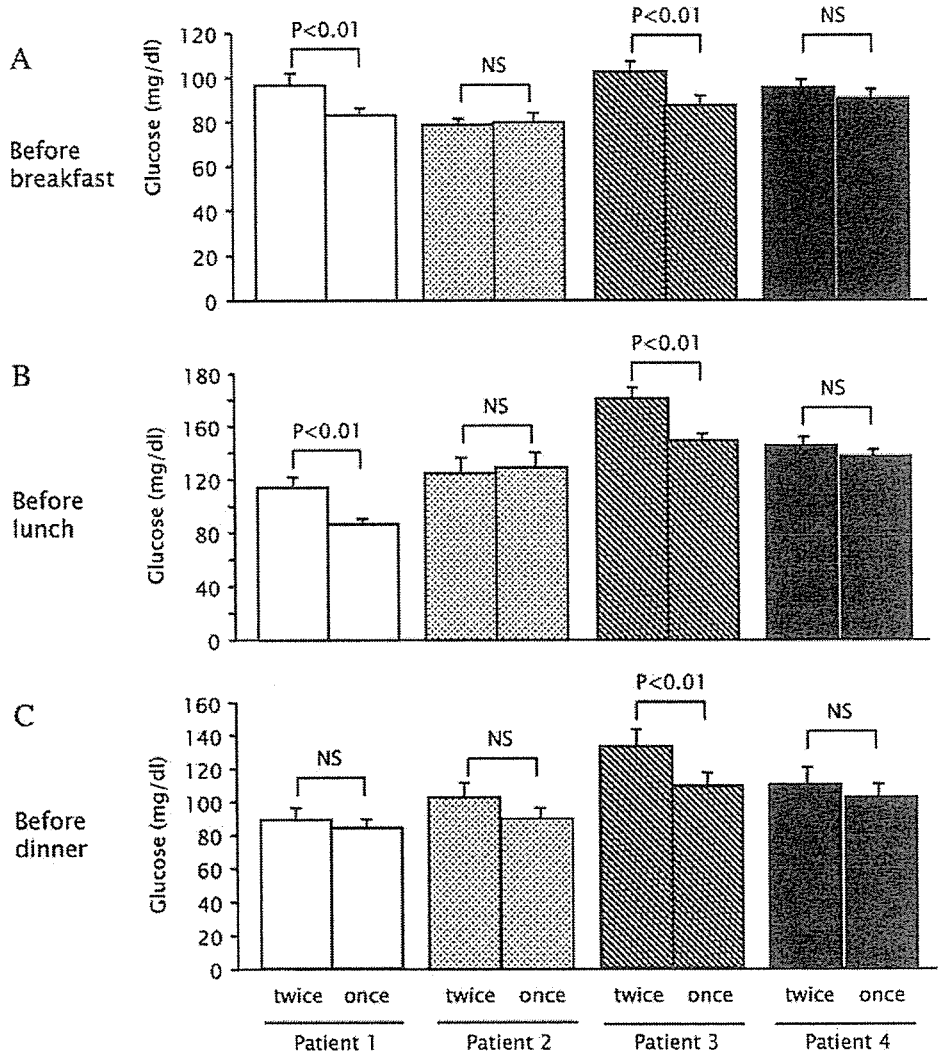

FIG. 3. Comparison of the mean (± SE) plasma glucose levels during the 12th month under the protocol of twice-daily leptin injection and during the 13th month under the protocol of once-daily leptin injection before breakfast (A), lunch (B), and dinner (C) in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), and patient 4 (*black bars*). NS, No significance difference ($P > 0.05$) between groups.

were well controlled throughout the therapy period (Fig. 2, C–F).

Antileptin antibodies

Patients 2 and 3, both CGL patients, showed elevations of basal plasma leptin levels, 75.0 and 42.4 ng/ml at the end of the 12th month, respectively. We detected antileptin antibodies in both patients. Antibodies from these patients did not neutralize the action of leptin at all in a bioassay.

Diabetes and other complications

All seven patients had normal renal functions at baseline; however, two patients had microalbuminuria (>30 mg/d), and two patients had macroalbuminuria (>300 mg/d) (Table 1). In addition, five of seven patients had elevated creatinine clearance (mean ± SE, 206.5 ± 22.0 ml/min·1.73 m^2) at the level above 125 ml/min·1.73 m^2. After the initiation of leptin-replacement therapy, urine albumin excretion of patients 1 and 3 with microalbuminuria began to decrease gradually within 1 month and was normalized within 2 months (Fig. 4A). Macroalbuminuria of patients 4 and 6 was also regressed to microalbuminuria within 3 and 1 month, respectively (Fig. 4B). In parallel, the creatinine clearance of the five patients with glomerular hyperfiltration significantly decreased to 129.5 ± 24.5 ml/min·1.73 m^2 (mean ± SE) for the 4-month leptin-replacement therapy ($P < 0.05$). These beneficial effects of leptin on urine albumin excretion and glomerular hyperfiltration were stable for up to 36 months.

Six of seven patients showed no diabetic retinopathy, but patient 7 had a nonproliferative retinopathy at baseline. No deterioration of her retinopathy was observed during the therapy. Six of seven patients had no diabetic neuropathy at baseline, although patient 6 showed neurogenic bladder. During the therapy period, her neurogenic bladder did not worsen, and no patients developed diabetic retinopathy or diabetic neuropathy.

Five of seven patients had moderate to severe acanthosis nigricans at baseline, but the acanthosis nigricans was improved in five patients after the leptin-replacement therapy.

Four of five female patients who were of reproductive age had hypogonadotropic amenorrhea at baseline as previously reported (26, 27) but resumed and sustained normal menses

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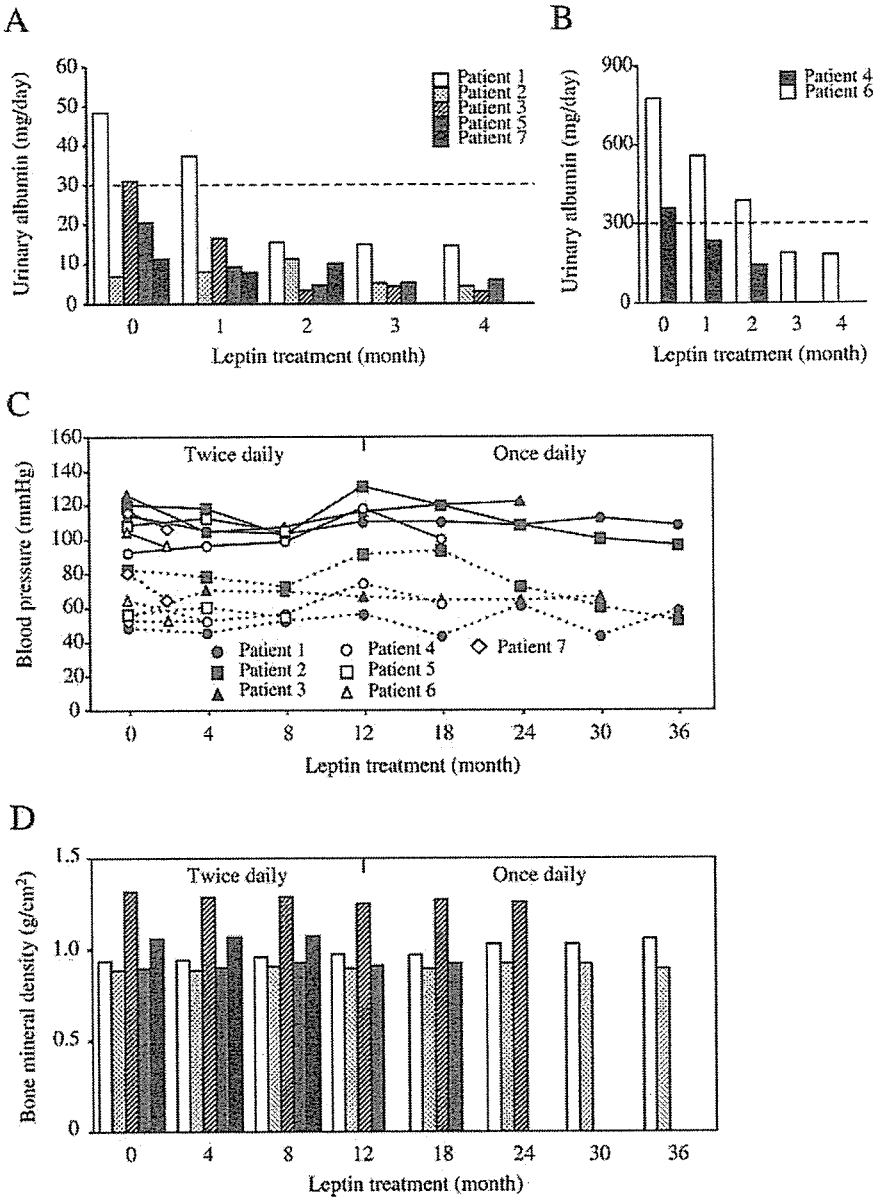

FIG. 4. A and B, Daily excretion levels of urinary albumin before and after 1, 2, 3, and 4 months of the leptin-replacement therapy in patients 1, 2, 3, 5, and 7 and in patients 4 and 6. C, Blood pressure before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in each patient. *Solid lines* indicate systolic blood pressure, and *broken lines* indicate diastolic blood pressure. D, Whole-body bone mineral density before and after 4, 8, 12, 18, 24, 30, and 36 months of the leptin-replacement therapy in patient 1 (*white bars*), patient 2 (*dotted bars*), patient 3 (*hatched bars*), patient 4 (*gray bars*), and patient 5 (*black bars*).

after the initiation of the leptin therapy. In an 11-yr-old girl, the menarche was observed after 12 months of the leptin therapy.

All the patients indicated an improvement in feeling of satisfaction after a meal within 1 or 2 d after the initiation of leptin therapy. This effect was sustained throughout the leptin therapy. For the first 4 months, a tendency of body weight reduction was observed in all the patients, but this change was not significant (mean ± SE, 40.9 ± 3.5 to 38.1 ± 3.1 kg, $P = 0.55$). After the first 4 months, the body weight was almost unchanged throughout the leptin therapy.

Adverse effects

We carefully observed blood pressure in the patients. At baseline, no patients showed hypertension (Table 1), and no distinct elevation of blood pressure was observed at any time throughout the therapy period (Fig. 4C).

No patients showed abnormal bone mineral density (Table 1). Whole-body bone mineral densities of the patients were unchanged for up to 36 months (Fig. 4D).

In all the patients, no other adverse effects of the leptin-replacement therapy including skin reactions at injection sites were detected for up to 36 months.

Discussion

In the present study, all of the leptin injections were done by medical doctors, because self-injection of leptin, which is not approved as a drug, is not permitted in Japan. In addition, all the patients were evaluated as inpatients during the initial 4 months of the leptin-replacement therapy. After leaving the hospital, the patients attended local clinics every day for every leptin injection. This allowed close supervision of leptin-replacement therapy, and the patients' lifestyles including diet and exercise were maintained constant. This

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condition could minimize the influences of compliance of leptin injection and changes of diet and exercise. Although we could not include a randomized, placebo-treated control group in the present study because of the rarity and clinical diversity of generalized lipodystrophy, it is highly likely that the improved metabolic control is due to the leptin therapy rather than to an improvement in general compliance associated with participation in the study.

In previous reports, improvements of glucose and triglyceride levels, glucose tolerance, and insulin sensitivity were reported at 1 month (18, 25, 28). The present study clearly shows that significant reductions of fasting glucose levels are achieved within 7 d after the initiation of the leptin-replacement therapy, and substantial reductions of the triglyceride levels are also gained within 7 d (Table 2). These rapid and powerful effects of leptin-replacement therapy were further confirmed with the glucose tolerance test and hyperinsulinemic-euglycemic glucose clamp study performed after 7 or 10 d in patient 4. These rapid effects on glucose and lipid metabolism in the present study are comparable to the rapid effects of leptin administration in two different mouse models of generalized lipodystrophy (16, 17).

After 12 months of the twice-daily leptin treatment, we tried to alter the leptin injection protocol to a once-daily injection without change of total daily dose. This protocol alteration did not affect the controls of glucose and lipid metabolism, and these controls were maintained for up to 24 months (Figs. 2 and 3). These observations demonstrate that a once-daily leptin injection is sufficient to control glucose and lipid metabolism in patients with generalized lipodystrophy.

In the present study, we detected antileptin antibodies in two of four tested patients. Both of them were CGL patients, whereas we did not detect antileptin antibodies in AGL patients. This observation raises the possibility that antileptin antibodies more easily develop in CGL patients than AGL patients. Antibodies from both of our CGL patients did not neutralize the leptin action *in vitro* bioassay. In at least one child with congenital leptin deficiency, the transient appearance of neutralizing antibodies against leptin was reported (21). It is possible to speculate that neutralizing antibodies against leptin more easily develop in patients with congenital leptin deficiency than CGL patients, who have a little leptin levels.

The leptin-replacement therapy substantially ameliorated or did not worsen diabetic complications. Amelioration of proteinuria in the present study is consistent with our and other's previous reports that leptin-replacement therapy significantly alleviates the glomerular injury and proteinuria of lipoatrophic diabetes in mice and humans (29, 30). Although we could not perform renal biopsies, it is highly likely that proteinuria observed in our patients is due to diabetic nephropathy because their proteinuria and hyperfiltration were evidently improved in parallel with the metabolic improvement. These findings indicate that leptin is useful to treat, at least, a certain type of diabetic nephropathy.

The leptin-replacement therapy did not induce elevation of blood pressure in any patients throughout the therapy period (Fig. 4C). We previously demonstrated that a high plasma leptin level that is 10 times of that in normal controls elevates blood pressure through the activation of the sympathetic nervous system in mice (31). It is highly likely that the leptin-replacement therapy at the physiological replacement dose does not affect blood pressure.

Bone mineral density of the patients was within normal range at baseline and was unchanged during the therapy period for up to 36 months (Fig. 4D), consistent with the study reported previously (32). We also previously demonstrated that leptin is a powerful inhibitor of bone formation in mice (33). Although the present study indicates that the leptin-replacement therapy at the physiological replacement dose does not affect bone mineral density in humans, careful follow-up is necessary for young patients.

The effect of leptin on β-cell function remains unclear. Leptin treatment decreased serum insulin levels in mouse models of lipodystrophy (16, 17) and human lipoatrophic patients in the United States (18). These decreases of insulin levels were explained by the reduction of glucose levels rather than the suppressive effect of leptin. Indeed, insulin levels peaked earlier in lipoatrophic patients in the United States, although their overall amounts of insulin secreted in response to the glucose load were less after the leptin therapy than at baseline (28). On the other hand, we here demonstrate that leptin-replacement therapy dramatically improves insulin secretion in Japanese AGL patients. Because glucose-lowering therapy often leads to the restoration of β-cell function in patients with diabetes, this effect can be explained at least in part by the cancellation of glucotoxicity (34). The different responses of insulin secretion to leptin-replacement therapy between AGL and CGL patients could be accounted for by the different duration of diabetes. The impaired insulin responses to the glucose load in CGL patients suggests that their β-cell functions were already exhausted before leptin-replacement therapy. Although whether leptin has an additional effect on β-cell is unknown, we here demonstrate that leptin-replacement therapy is beneficial to the treatment of impaired β-cell function.

The mechanisms through which leptin exerts its insulin-sensitizing actions are unclear at present. Fat accumulation in the insulin target organs, which causes so-called lipotoxicity, is considered to be one of the mechanisms for insulin resistance in patients with lipodystrophy (35). Because in patient 4 with AGL, the improvement of insulin sensitivity was observed before a substantial decrease of tissue lipid content in the liver and muscle, additional studies are necessary to clarify the relationship between insulin resistance and the tissue lipid content in humans.

Based on the effect of the leptin-replacement therapy, it is highly likely that leptin deficiency is the main cause of the metabolic abnormalities associated with lipodystrophy. However, the adipose tissue is recognized as the largest endocrine organ. Therefore, it is possible to speculate that these hormones other than leptin may be involved to some degree in the pathogenesis of lipoatrophic diabetes.

Using leptin-overexpressing transgenic skinny mice (8), we previously reported that leptin treatment is useful for treatment of not only lipoatrophic diabetes mice (17) but also other diabetic mice models (36, 37). These observations along with dramatic effects and safety of the leptin therapy in the

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present study indicate possible application of the leptin therapy to diabetes and its complications.

In summary, under strict control of lifestyle and an extremely high compliance of leptin injection, we demonstrate that the leptin-replacement therapy improves both insulin sensitivity and insulin secretion dramatically and rapidly improves glucose and lipid metabolism in patients with generalized lipodystrophy, and its effects are maintained for up to 36 months without any adverse effects. In addition, the leptin-replacement therapy is beneficial to diabetic complications and lipodystrophic ones. The once-daily leptin injection is sufficient to control glucose and lipid metabolism for a long time. It is concluded that leptin-replacement therapy is an effective and safe treatment for long-term improvement of glucose and lipid metabolism and complications in generalized lipodystrophy.

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Safety and Efficacy of Low-dose Pioglitazone (7.5 mg/day) vs. Standard-dose Pioglitazone (15 mg/day) in Japanese Women with Type 2 Diabetes Mellitus

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Abstract. It is well known that pioglitazone, a potent thiazolidinedione, improves metabolic control. However, weight gain or peripheral edema may be of major clinical concern when using this agent. The purpose of our study was to prospectively evaluate the effects of low-dose pioglitazone (7.5 mg/day) on metabolic control, weight gain and the incidence of edema compared with a standard dose of pioglitazone (15.0 mg/day) in patients with type 2 diabetes mellitus (T2DM). Ninety-five Japanese female patients (mean age 58.4 ± 10.4 years) with newly diagnosed T2DM were selected for this study. They were randomly divided into the following 2 groups according to therapy regimens, and examined every month for 6 months after diagnosis. Group A consisted of 54 patients treated with low-dose pioglitazone orally; Group B, the control-group, consisted of 41 patients treated with standard-dose pioglitazone orally. The incidence of peripheral edema was significantly much lower in group A (2/54) than in group B (11/41) ($p = 0.0014$). In addition, % change of body weight during the 6-month treatment in group A was significantly less than that in group B ($p<0.0001$). On the other hand, the % change of biochemical parameters including HbA1c did not differ significantly between group A and group B, although glucose and lipid control significantly improved from baseline in both groups. Our results demonstrate the safety and efficacy of low-dose pioglitazone, suggesting that it could be another good choice of treatment for Japanese women with T2DM.

Key words: Pioglitazone, Thiazolidinedione, Diabetes mellitus

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IT is well known that pioglitazone, a member of the thiazolidinedione (TZD) class, improves glycemic control, reduces hyperinsulinemia and improves lipid metabolism, mainly by activating peroxisome proliferator-activated receptor-γ (PPAR-γ) [1–5]. Moreover, because the antiatherogenic effects of TZDs are likely to be mediated through their direct action on the vasculature, pioglitazone may confer benefits beyond decreases in glucose level [1–7]. However, certain clinical concerns make clinicians hesitant to use it in Japan. One of such concerns is liver toxicity, since troglitazone, the first agent in this class to become available for clinical use in Japan, was withdrawn due to reports of severe hepatic injury [2–5]. However, we should not be overly concerned about the hepatotoxicity of pioglitazone, provided that liver function tests are performed at baseline and periodically thereafter [2–6]. Indeed, there have been a few reports of hepatotoxicity potentially caused by pioglitazone [4, 8]. In these reports, however, the agent was never proved to be the cause of the hepatotoxicity, and the condition was resolved with supportive care and withdrawal of the putative agent [4, 8].

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On the other hand, fluid retention, weight gain, or peripheral edema may be of major clinical concern, because these are the most frequent adverse events associated with pioglitazone [2–6, 9], occurring more often in women than in men [6]. The incidence of edema is reported to be about 5–10%, although the incidence rises to 13–16% when combined with insulin therapy [4, 6, 9–15]. Fluid retention is also reported to account for a 6%–7% increase in plasma volume [4, 9]; thus pioglitazone is contraindicated in patients with severe congestive heart failure (CHF), defined as New York Heart Association (NYHA) functional class III and IV [2–5, 9]. Weight gain partly due to fluid retention is reported to be significant and dose-dependent (ranging 0.5–4.5 kg) [4, 10, 14, 15]. These frequent and undesirable effects of pioglitazone, even if not fatal, may lead to the breakdown of the doctor-patient relationship and consequently discontinuation of treatment of type 2 diabetes mellitus (T2DM) itself. In order to reduce these adverse effects of pioglitazone, we tried a lower dosage on patients with T2DM.

The aim of our study was to prospectively evaluate the effects of low-dose pioglitazone (7.5 mg/day) on glucose and lipid metabolism, weight gain and the incidence of edema compared with a standard-dose of pioglitazone (15.0 mg/day) in Japanese women with T2DM.

Materials and Methods

Subjects

Ninety-five Japanese female patients (mean age 58.4 ± 10.4 years) with newly diagnosed T2DM who initially attended the clinic of Rakuwakai Otowa Hospital during the period between November 2003 and October 2004, were selected for this study. The diagnosis of T2DM was established on the basis of abnormal glucose tolerance test results, classic symptoms, and laboratory findings. The patients were randomly divided into the following 2 groups according to therapy regimen, and examined every month for 6 months after diagnosis. Group A consisted of 54 patients treated with low-dose pioglitazone (7.5 mg/day) orally; Group B, the control-group, consisted of 41 patients treated with standard-dose pioglitazone (15.0 mg/day) orally. Their clinical data are shown in Table 1.

All subjects were instructed to adhere to a disease- and weight-oriented diet and exercise regimen throughout the study. Dietary and exercise advice was given at baseline and every month after the diagnosis during the 6-month treatment, with the target of body weight normalization and supply of individually appropriate calories and nutrients. All subjects were examined by the

Table 1. Mean ± SD of the variables assessed in the examined subjects

	Group A (n = 53) (pioglitazone 7.5 mg/day)		Group B (n = 31) (pioglitazone 15 mg/day)	
	Baseline	6 months later	Baseline	6 months later
Age (years)	57.85 ± 10.8	58.29 ± 10.9	59.13 ± 10.0	59.51 ± 10.1
BMI (kg/m^2)	23.86 ± 2.6	24.49 ± 3.0	24.21 ± 2.7	25.46 ± 2.8
Height (cm)	155.76 ± 8.9	155.16 ± 9.1	155.24 ± 9.4	154.96 ± 9.5
Weight (kg)	57.95 ± 8.0	59.09 ± 9.2	58.69 ± 10.8	61.48 ± 11.1
SBP (mmHg)	129.4 ± 14.2	126.7 ± 15.5	130.6 ± 13.1	125.2 ± 13.9
DBP (mmHg)	71.5 ± 9.7	68.8 ± 10.6	72.0 ± 9.9	68.3 ± 10.4
FPG (mg/dL)	169.25 ± 22.8	145.34 ± 25.1†	171.97 ± 22.4	144.1 ± 25.1†
HbA1c (%)	7.57 ± 1.0	6.96 ± 1.0†	7.69 ± 0.9	7.00 ± 1.1†
IRI (μU/mL)	8.04 ± 2.9	7.33 ± 3.7†	7.88 ± 3.0	6.94 ± 3.7†
HOMA-IR	3.31 ± 0.4	2.64 ± 0.5†	3.36 ± 0.4	2.47 ± 0.4†
TC (mg/dL)	218.17 ± 27.2	216.94 ± 28.8	215.84 ± 27.4	213.77 ± 31.5
TG (mg/dL)	177.57 ± 38.3	156.25 ± 52.7*	172.32 ± 37.9	145.39 ± 28.3†
HDL (mg/dL)	45.64 ± 8.5	49.17 ± 8.2*	44.29 ± 8.8	48.84 ± 8.8*
LDL (mg/dL)	137.02 ± 16.2	136.52 ± 18.4	137.08 ± 17.2	135.86 ± 18.0

BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; FPG, fasting plasma glucose; IRI, immunoreactive insulin; HOMA-IR, homeostasis model assessment for insulin resistance; TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol.

* $p<0.05$, † $p<0.01$ vs. baseline values. None of the intergroup differences reached statistical significance.

doctor as to their general health, body weight, systolic and diastolic blood pressure (SBP and DBP), symptoms and signs suggestive of adverse reactions, and compliance with the medication, dietary therapy, and exercise therapy, at baseline and every month after the diagnosis during the 6-month treatment. BP was measured with the patient in sitting position after at least 5 minutes of rest.

We excluded subjects who had a history of glucose-lowering pharmacotherapy or corticosteroids, and specific contraindications to pioglitazone including increased serum levels of liver enzyme (aspartate aminotransferase [AST] or alanine aminotransferase [ALT] >2.5 times the upper limit of normal) and CHF defined as NYHA class III or IV. At study entry, antihypertensive agents except β-blockers and thiazides, and lipid-lowering agents, if administered for more than 3 months, were also allowed, provided that the dose was not changed during this study. None of the subjects was a heavy smoker or alcoholic.

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethical Committee of Rakuwakai Otowa Hospital. All the subjects gave their informed consent before they were enrolled.

Biochemical measurements

All subjects underwent laboratory blood tests at baseline, and every month after the diagnosis during the 6-month study period. Serum samples were obtained before 8:00 AM after an overnight fast, and were immediately processed and kept frozen at –20°C until the assays were carried out. Serum AST, ALT, fasting plasma glucose (FPG), total cholesterol (TC), triglycerides (TG), and high density lipoprotein cholesterol (HDL) were measured using routine laboratory methods. Low density lipoprotein cholesterol (LDL) was calculated by Friedewald equation (LDL = TC – [HDL + TG/5]). HbA1c was measured by the latex agglutination method (Fujirebio, Inc., Tokyo, Japan). Plasma insulin concentration (immunoreactive insulin [IRI]) was measured by enzyme immunoassay using a commercially available kit (Tosoh, Tokyo, Japan). The insulin resistance index was assessed by the homeostasis model assessment for insulin resistance (HOMA-IR).

Statistical analysis

Data were analyzed by the Student's unpaired t-test and paired t-test to assess intergroup differences and the longitudinal differences in each group, respectively, and by χ^2-test to assess intergroup differences of incidence. Statistics were calculated with Stat View version 5.0 (Abacus Concepts, Inc., Berkeley, CA). A P value <0.05 was considered as statistically significant.

Results

Table 1 shows the baseline characteristics of patients in group A and group B. There was no significant difference between the two groups in terms of age, height, weight, body mass index (BMI), SBP, DBP, and biochemical parameters including TC, TG, HDL, FPG, HbA1c, and IRI at baseline.

Mild peripheral edema occurred in 2 patients in group A and 11 patients in group B during this study. Among them 1 patient in group A and 10 in group B dropped out of the study. However, in these 13 patients, ultrasonography did not show any abnormalities, and peripheral edema was resolved spontaneously with cessation of pioglitazone. The incidence of peripheral edema was significantly much lower in group A than in group B ($p = 0.0014$).

Body weight increased, but not significantly, from baseline during the treatment period in both groups (Table 1, Fig. 1). However, % change of body weight in group A was significantly less than that in group B ($p<0.0001$) (Fig. 2).

Both SBP and DBP were lower than at baseline, but not significantly so, after treatment (Table 1). The % change of BP was also not different between group A and group B.

FPG, HbA1c, IRI, and HOMA-IR significantly improved from baseline during 6-month treatment in both groups (Table 1, Fig. 1). The % change of these parameters did not differ significantly between group A and group B (Fig. 2).

While TC and LDL hardly improved, TG and HDL significantly improved from baseline during 6-month treatment in both groups (Table 1, Fig. 1). The % change of these parameters did not differ significantly between group A and group B (Fig. 2).

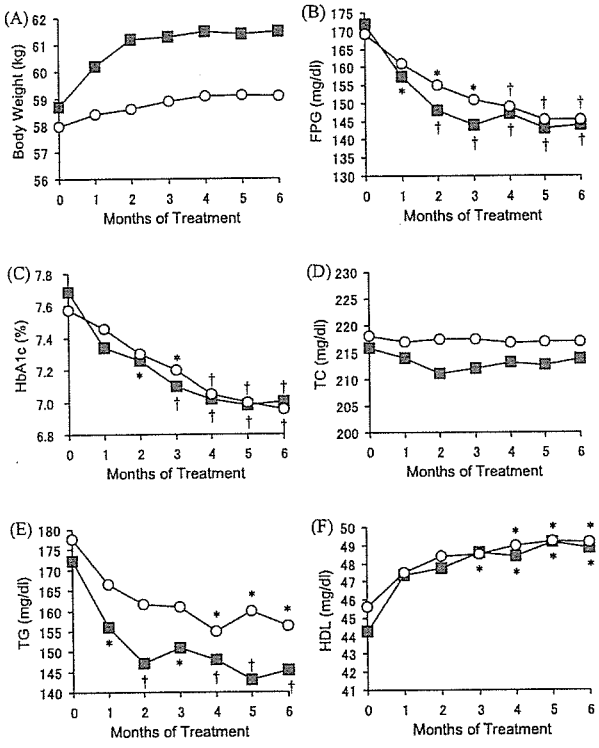

Fig. 1. Body weight (A), FPG (B), HbA1c (C), TC (D), TG (E), and HDL (F) (mean values) during the 6-month treatment in patients of group A (white circles) and group B (black squares). * $p<0.05$, † $p<0.01$ vs. baseline values. None of the intergroup differences reached statistical significance.

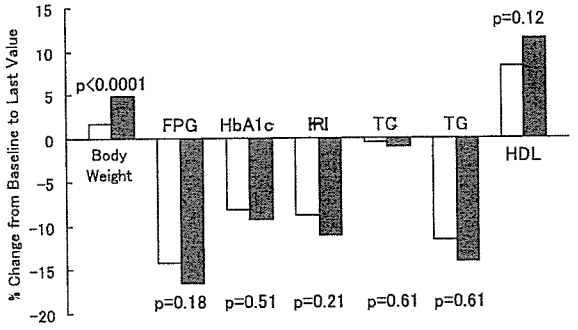

Fig. 2. Changes in body weight, FPG, HbA1c, IRI, and lipid profiles from baseline to last value in patients of group A (white bars) and group B (black bars). *P*-values for comparisons between group A and group B.

Discussion

In the present study of low-dose pioglitazone in Japanese women with T2DM, we found that low-dose pioglitazone (7.5 mg/day) had a comparable benefit on glucose and lipid metabolism, compared with standard-dose pioglitazone (15 mg/day). On the other hand, we also found that its deleterious effects, including weight gain and peripheral edema, were significantly more modest in low-dose pioglitazone. These results suggest that, considering its efficacy, safety, and cost-effectiveness, low-dose pioglitazone may be preferred for treating Japanese women with T2DM compared with the standard-dose.

Previous studies in rats revealed that pioglitazone dose-dependently reduced hyperglycemia, hyperlipidemia, and hyperinsulinemia [16]. Since then, it has been clinically described that the effects of pioglitazone on glycemic control are dose-dependent [3, 4, 10, 14]. We do not deny these dose-dependent effects of pioglitazone, because responsiveness to pioglitazone was found to be relatively better in the 15-mg group than in the 7.5-mg group in our study, although not significantly so.

Recently, Aronoff *et al.* [10] evaluated the efficacy and safety of four doses of pioglitazone monotherapy in the treatment of patients with T2DM in a large-scale multicenter trial. While their results showed a dose-dependent beneficial effect of pioglitazone on glycemic control in T2DM, its effect on glycemic control was comparable between the 15-mg group and the 30-mg group. The baseline characteristics in their study were somewhat different from those in ours. While all the patients in our study are Japanese and female, and had never received pharmacological antidiabetic therapy, those in their study were racially-mixed, included both men and women, and only 31% of them were naive to prior antidiabetic therapy. Another difference is that the baseline HbA1c and FPG were relatively higher in their study (>10.0% and >260 mg/dL, respectively) than in our study. It has been suggested in the literature that the magnitude of the response to pioglitazone should be greater in those naive to prior antidiabetic therapy and in those with better metabolic control [4, 5], as Aronoff *et al.* stated in their report [10]. Therefore, the response to low dose pioglitazone may have been greater in our study due to earlier diagnosis or better metabolic control at the onset.

More recently, Miyazaki *et al.* [14] also investigated the dose-response effects of pioglitazone on glycemic control in patients with T2DM. While the dose-dependent beneficial effects of pioglitazone on glycemic control were shown also in their study, the improve-

ment of FPG in the 7.5-mg group was superior to that in the 15-mg group. Furthermore, considering that the baseline HbA1c in their study had been substantially higher in the 7.5-mg group than in the 15-mg group, it can be said that the 15-mg group had a potential advantage over the 7.5-mg group in responding to pioglitazone and, therefore, that the improvement of HbA1c was comparable between the 7.5- and 15-mg group in their study. Thus, their results are supportive of ours which showed comparable effects on glycemic control between the 7.5-mg group and the 15-mg group.

In the Japanese literature, there have been several studies showing that the effect of 15-mg pioglitazone administered every other day on glycemic control is comparable with that of 15-mg pioglitazone everyday in patients with T2DM [17–19]. Another study described that reducing dose of pioglitazone from 15 mg/day to 7.5 mg/day did not alter metabolic control in Japanese patients with T2DM (3 men and 8 women) [20]. These findings are compatible with our results, indicating that 7.5-mg pioglitazone is potentially useful in improving glycemic control.

It has been proposed that TZDs including pioglitazone may correct other metabolic abnormalities, such as dyslipidemia and arterial hypertension [1–6, 10, 14, 15, 21–24], as found also in our study. In a study examining Japanese patients with T2DM, a statistically significant increase of HDL from the baseline was found with 7.5-mg pioglitazone [25], consistent with our results. These beneficial effects of pioglitazone are considered to be mainly attributed to the improvement of insulin resistance [1–6]. In the study by Miyazaki *et al.* [13] reduction in IRI was comparable among the subgroups treated with different doses of pioglitazone, and 7.5-mg pioglitazone substantially decreased IRI (means ± SEM) from 25 ± 5 μU/mL to 20 ± 3 μU/mL during the 26-week treatment period. Their findings were consistent with ours, suggesting the beneficial effects of 7.5-mg pioglitazone.

The severity of weight gain and peripheral edema due to pioglitazone has also been reported to be dose-dependent [4, 10, 14], as seen in our study. A considerably high percentage (26.8%) of our patients treated with 15-mg pioglitazone did experience edema, although mild, and they refused to take pioglitazone any longer even if its dose was reduced. However, weight gain and edema were almost negligible in our patients treated with 7.5-mg pioglitazone, suggesting the safety of 7.5-mg pioglitazone. Additionally, consistent with previous reports [2–6, 10, 14, 15], there were no cases of drug-induced hepatotoxicity or hypoglycemia in either group in our study, while hypoglycemia is a common adverse event in other glucose-lowering agents [26].

In conclusion, we showed that 7.5-mg pioglitazone significantly improved glucose and lipid metabolism, and, in addition, it was well tolerated in this study. Our results demonstrate the safety and efficacy of 7.5-mg pioglitazone per day, suggesting that low-dose pioglitazone (7.5 mg/day) could be another good choice of treatment for Japanese women with T2DM.

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Adrenomedullin/Cyclic AMP Pathway Induces Notch Activation and Differentiation of Arterial Endothelial Cells From Vascular Progenitors

Takami Yurugi-Kobayashi, Hiroshi Itoh, Timm Schroeder, Akiko Nakano, Genta Narazaki, Fumiyo Kita, Kentoku Yanagi, Mina Hiraoka-Kanie, Emi Inoue, Toshiaki Ara, Takashi Nagasawa, Ursula Just, Kazuwa Nakao, Shin-Ichi Nishikawa, Jun K. Yamashita

Objective—The acquisition of arterial or venous identity is highlighted in vascular development. Previously, we have reported an embryonic stem (ES) cell differentiation system that exhibits early vascular development using vascular endothelial growth factor (VEGF) receptor-2 (VEGFR2)-positive cells as common vascular progenitors. In this study, we constructively induced differentiation of arterial and venous endothelial cells (ECs) in vitro to elucidate molecular mechanisms of arterial-venous specification.

Methods and Results—ECs were induced from VEGFR2$^+$ progenitor cells with various conditions. VEGF was essential to induce ECs. Addition of 8bromo-cAMP or adrenomedullin (AM), an endogenous ligand-elevating cAMP, enhanced VEGF-induced EC differentiation. Whereas VEGF alone mainly induced venous ECs, 8bromo-cAMP (or AM) with VEGF supported substantial induction of arterial ECs. Stimulation of cAMP pathway induced Notch signal activation in ECs. The arterializing effect of VEGF and cAMP was abolished in recombination recognition sequence binding protein at the Jκ site deficient ES cells lacking Notch signal activation or in ES cells treated with γ-secretase inhibitor. Nevertheless, forced Notch activation by the constitutively active Notch1 alone did not induce arterial ECs.

Conclusions—Adrenomedullin/cAMP is a novel signaling pathway to activate Notch signaling in differentiating ECs. Coordinated signaling of VEGF, Notch, and cAMP is required to induce arterial ECs from vascular progenitors. (*Arterioscler Thromb Vasc Biol.* **2006;26:1977-1984.**)

Key Words: angiogenesis ■ developmental biology ■ embryonic stem cells ■ endothelium ■ vascular biology

Vascular formation is a complicated but well-organized process that involves sprouting, branching, and differential growth of vessels from the primary plexus or existing vessels into a functioning circulation system.[1] During the process, vascular cell specification proceeds in an inseparably coordinated manner.[2] A transmembrane ligand, ephrinB2, and its receptor, the tyrosine kinase EphB4, are reported as molecular markers for arterial and venous endothelial cells (ECs), respectively.[3,4] Recently, various molecular markers specific for arterial ECs have been documented such as Delta-like 4 (Dll4), Bmx, Notch1, Activin receptor-like kinase 1 (Alk1), and others.[5,6] These findings enable the investigation of endothelial specification processes at the cellular and molecular levels being independent of the context of vessel location within the body plan.

The Notch pathway has been highlighted in arterial-venous specification.[7,8] Notch target genes, Hairy and Enhancer-of-split-related basic helix-loop-helix transcription factors, such as grl (gridlock) in zebrafish, or Hey1 and 2 in mammals, are required for arterial vascular development.[9,10] Arterial-venous specification mechanisms in zebrafish were further demonstrated to be a regulatory signaling cascade of sonic hedgehog-vascular endothelial growth factor (VEGF)-Notch-ephrinB2.[5] The molecular machinery for arterial-venous specification in mammals, however, is still undergoing investigation.

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cAMP is a ubiquitous second messenger produced in cells and is involved in various biological phenomena including cell growth and differentiation.[11] Nevertheless, little has been reported for the role of cAMP signaling in vascular development. Adrenomedullin (AM) is a multifunctional polypeptide that was originally isolated from human pheochromocytoma.[12] AM exerts its function by increasing the levels of

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intracellular cAMP through the binding to its receptor complex, calcitonin receptor-like receptor (CRLR), and receptor activity modifying proteins (RAMP)-2 or RAMP-3.[13] Targeted null mutation of the AM gene shows embryonic lethality[14] with aberrant vascular formation and hemorrhage,[15] or extreme hydrops fetalis and cardiovascular abnormalities, including underdeveloped arterial walls,[16] inferring the significance of AM/cAMP signaling in vascular development.

Pluripotent embryonic stem (ES) cells are potent materials for both regenerative therapeutic approaches and developmental research. We have developed a novel ES cell differentiation system devoid of embryoid body formation or feeder cells that exhibits early vascular development using VEGF receptor-2 (VEGFR2)-positive cells as common progenitors for vascular cells.[17,18] We demonstrated that ES cell-derived VEGFR2$^+$ cells can differentiate into both ECs and mural cells (MCs) (pericytes and vascular smooth muscle cells) and form mature vascular-like structures in vitro.[18] Moreover, transplantation of induced vascular cells can augment the blood flow in tumor angiogenesis.[19] Our ES-derived VEGFR2$^+$ cell differentiation system can recapitulate the vascular development processes and dissect the cellular and molecular mechanisms of each developmental step including endothelial differentiation and specification.

In this study, we aimed to specifically induce arterial and venous ECs and elucidate the mechanisms of arterial-venous specification using our ES cell differentiation system. We successfully induced arterial and venous ECs and demonstrated that the AM/cAMP pathway is another indispensable signaling pathway in EC differentiation and arterial specification in conjunction with VEGF and Notch by reconstructing the arterial EC differentiation process in vitro. Our constructive approach using this ES cell system provides a novel understanding of the cellular and molecular mechanisms of vascular developmental processes.

Methods

Antibodies

Monoclonal antibodies for murine E-cadherin (ECCD2), murine VEGFR2 (AVAS12), and murine VE-cadherin (VECD1) were described previously.[18] Monoclonal antibodies for murine CD31 and CXCR4 were purchased from Pharmingen (San Diego, Calif). MoAb for murine alpha smooth muscle actin (SMA) 1A4 and human estrogen receptor-α (ERα) (F-10) antibody were from Sigma (St Louis, Mo) and Santa Cruz Biotechnology (Santa Cruz, Calif), respectively. Cleaved Notch1 antibody was from Cell Signaling Technology (Beverly, Mass).

Cell Culture

Induction of differentiation of an ES cell line, CCE (gift from Dr Evans), were performed using differentiation medium (alpha minimal essential medium; Gibco, Grand Island, NY) supplemented with 10% fetal calf serum (Equitech-Bio, Kerrville, Tex) and 5×10^{-5} mol/L 2-mercaptoethanol (Gibco) and VEGF165 (R&D System, Minneapolis, Minn) as previously described.[17,18] Other chemicals, rat AM (Peptide Institute. Inc, Osaka, Japan), 8-bromoadenosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cAMP) (Nacalai Tesque, Kyoto, Japan), 8-bromoguanosine-3′:5′-cyclic monophosphate sodium salt (8bromo-cGMP) (Nacalai Tesque), 3-isobutyl-1-methyl-xanthine (IBMX) (Nacalai Tesque), or γ-secretase inhibitor IX, DAPT (Calbiochem, San Diego, Calif), and iloprost (Cayman Chemical, Ann Arbor, Mich) were occasionally added to VEGFR2$^+$ cell culture.

The recombination recognition sequence binding protein at the Jκ site (RBP-J$^{+/+}$), RBP-J$^{+/-}$ and RBP-J$^{-/-}$ D3 ES cell lines have been described previously.[20] The ES cell line NERT$^{\Delta O}$-7[21] was generated by stable introduction of CAG promoter-driven cDNA encoding a fusion protein of a constitutively active part of the intracellular domain of mouse Notch1 and a tamoxifen-sensitive mutant of the hormone binding domain of the human estrogen receptor α (NERT)[22] into EB5 ES cells (gift from Dr Niwa). To induce Notch activation, 4-hydroxytamoxifen (OHT) (50 to 500 nmol/L) (Sigma) was added to NERT$^{\Delta O}$-7 cell-derived VEGFR2$^+$ cells 12 hours after the plating. NERT$^{\Delta O}$-7/Hes-green fluorescent protein (GFP) cells were generated by stable introduction of Hes promoter-driven enhanced GFP (EGFP) gene[23] (gift from Dr Kageyama) into NERT$^{\Delta O}$-7 cells.

Flowcytometry and Cell Sorting

Fluorescence-activated cell sorting (FACS) of ES cells was performed as previously described.[17,18]

Immunocytochemistry

Immunostaining for cultured cells was performed as described.[18,24] Double immunofluorescent staining for CD31 and ERα was performed using anti-ERα antibody (1:50) and anti-CD31 antibody (1:300) as first antibodies, followed by second antibodies, Alexa Fluor 546-conjugated goat anti-rat IgG (1:500) and Alexa Fluor 488-conjugated goat anti-mouse IgG (1:500) (Molecular Probes, Eugene, Ore). For double staining for ephrinB2 and CD31, the fixed culture slides were incubated with EphB4-human immunoglobulin Fc portion chimeric protein (EphB4-Fc) (1:50; R&D system), followed by peroxidase-conjugated goat IgG fraction to human IgG Fc (1:500; ICN Biomedicals, Inc, Aurora, Ohio). TSA Biotin system (Tyramid signal amplification; PerkinElmer Life Science, Boston, Mass) was used for amplification of the signal for EphB4-Fc staining. EphrinB2$^+$ cells were visualized by using streptavidin-Alexa Fluor488-conjugate (Molecular Probes). Phycoerythrin-conjugated anti-CD31 antibody (Pharmingen) and DAPI (Molecular Probes) were added together with streptavidin-conjugated alexa 488. Cleaved intracellular domain of Notch (NICD) staining was performed using TSA Biotin System (PerkinElmer) with cleaved Notch1 antibody (1:300), followed by peroxidase-labeled anti-rabbit IgH (1:250; Vector Laboratories, Burlingame, Calif).

Single-Cell Analysis

Single-cell sorting of VEGFR2$^+$ cells using 96-well dishes was performed as previously described.[18] Colonies were stained for ephrinB2 using EphB4-Fc by TSA kit with streptavidin-conjugated horseradish peroxidase, followed by addition of phycoerythrin-conjugated anti-CD31 antibody and DAPI. Numbers of colonies including CD31$^+$ cells (EC-including), colonies including ephrinB2$^+$ cells (arterial EC-including), and ephrinB2$^+$ arterial EC numbers in each arterial EC-including colonies, as well as the total number of colonies that appeared were counted. 1692 VEGFR2$^+$ cells were cultured with VEGF alone, and 1128 cells were cultured with VEGF and 8bromo-cAMP. Total colony numbers in every 100 sequential wells, EC-including or arterial EC-including colony numbers in every 10 sequential colonies that appeared, and the arterial EC number in each arterial EC-including colony were statistically evaluated.

Measurement of Intracellular cAMP

After 3 days culture of VEGFR2$^+$ cells (2 to 10×10^5 cells), cells were harvested and counted. Intracellular cAMP concentration in total harvested cells was evaluated using cAMP Biotrak Enzyme Immunoassay system kit (Amersham Bioscience). Concentration was normalized by cell number.

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In Situ Hybridization

In situ hybridization for CXCR4 was performed as previously described.[25]

Reverse-Transcription Polymerase Chain Reaction Amplification

Total RNA was isolated from sorted VE-cadherin$^+$ ECs induced by VEGF alone, or 8bromo-cAMP and VEGF treatment, using ISOGEN (Nippon Gene, Toyama, Japan). The reverse-transcription polymerase chain reaction was performed as described[24] using indicated primers (supplemental Table I, available online at http://atvb.ahajournals.org).

Statistical Analysis

Statistical analysis of the data was performed using Student t test. $P<0.05$ was considered significant.

Results

We first examined the effects of AM and cAMP on EC differentiation from ES cell-derived VEGFR2$^+$ progenitor cells. VEGFR2$^+$ cells were sorted by FACS and re-cultured for 3 days on type IV collagen-coated dishes in differentiation medium (see Methods) with VEGF (50 ng/mL) and other factors. Double immunostaining of induced cells with an EC marker, CD31, and a MC marker, SMA, revealed that VEGF treatment selectively induced both CD31$^+$ ECs and SMA$^+$ MCs from VEGFR2$^+$ cells as previously reported[18] (Figure 1A). Simultaneous stimulation of cAMP signaling in the presence of VEGF substantially enhanced EC induction from VEGFR2$^+$ cells (Figure 1B to 1D). VEGF together with 0.5 mmol/L 8bromo-cAMP resulted in substantial induction of ECs (Figure 1D), whereas 8bromo-cAMP treatment alone exerted almost no effect (data not shown). Another cyclic monophosphate analog, 8bromo-cGMP, showed no effect on VEGF-induced EC induction (data not shown). Addition of 10^{-6}mol/L AM also enhanced VEGF-stimulated EC induction, but to a lesser extent than 8bromo-cAMP (Figure 1B). Enhancement of the effect of AM by the simultaneous administration of a phosphodiesterase inhibitor, IBMX, revealed comparable EC induction with 8bromo-cAMP (Figure 1C). We quantitatively evaluated the EC-inducing effects of AM and 8bromo-cAMP using flow cytometry. VEGF treatment induced ECs to ≈30% of total cells. AM increased VEGF-induced ECs up to ≈50%. AM with IBMX or 8bromo-cAMP showed efficient induction of ECs to ≈70% of total cells (Figure 1E). Intracellular concentration of cAMP in the differentiating cells was significantly increased by AM with VEGF (667.6 fmol±215.1/10^6 cells; n=6; $P<0.01$ versus VEGF alone), or AM and IBMX with VEGF (1142 fmol±270.1/10^6 cells; n=6; $P<0.001$ versus VEGF alone) than that with VEGF alone (372.2 fmol±58.5/10^6 cells; n=6), and was comparable or lower level with those observed in previous reports using human umbilical vein ECs.[26] These results indicated that the AM/cAMP pathway specifically and synergistically enhances the effect of VEGF on EC differentiation from VEGFR2$^+$ progenitor cells.

Next, we investigated the features of induced ECs with AM/cAMP treatment with regard to arterial-venous diversity. Arterial ECs were evaluated by ephrinB2 expression, an arterial EC marker, detected by the binding of EphB4-Fc.[27]

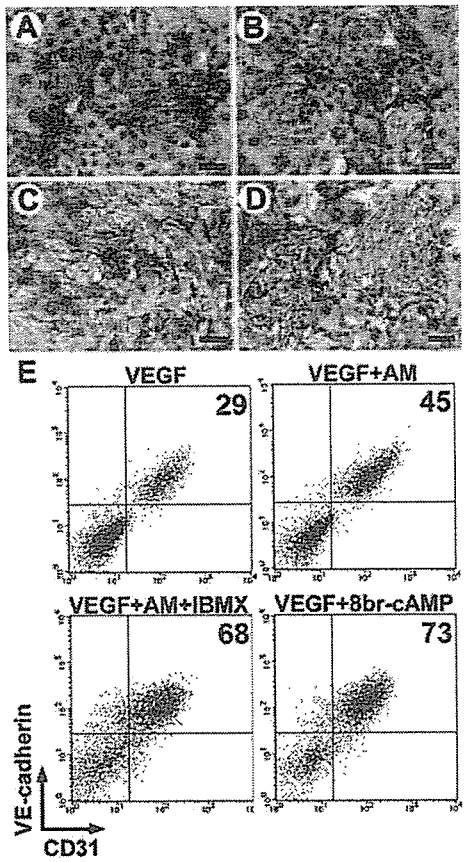

Figure 1. The effect of AM and cAMP on EC induction from VEGFR2$^+$ cells. A to D, Double immunostaining of induced ECs and MCs with an EC marker CD31 (purple) and MC marker SMA (brown) after 3 days of culture of VEGFR2$^+$ cells on type IV collagen-coated dishes in various conditions. A, VEGF treatment alone (50 ng/mL). CD31$^+$ EC sheets and SMA$^+$ MCs appear. B, VEGF with 10^{-6} mol/L AM. A slight increase of ECs is observed. C, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. D, VEGF with 0.5 mmol/L 8bromo-cAMP. Remarkable EC induction occurs. Scale bars: 100 μm. E, Flow cytometry of induced cells from VEGFR2$^+$ cells with endothelial markers VE-cadhein and CD31. Left upper panel, VEGF treatment alone (50 ng/mL). Right upper panel, VEGF with 10^{-6} mol/L AM. Left lower panel, VEGF with 10^{-6} mol/L AM and 10^{-4} mol/L IBMX. Right lower panel, VEGF with 0.5 mmol/L 8bromo-cAMP. Percentages of VE-cadherin$^+$/CD31$^+$ ECs of total VEGFR2$^+$ cell-derived cells are indicated.

We double-immunostained ECs using anti-CD31 antibody and EphB4-Fc (Figure 2A to 2D). With VEGF treatment alone, very few ephrinB2$^+$ arterial ECs were observed among the ECs that appeared, indicating that venous ECs were mainly induced in this condition (Figure 2A). Surprisingly, remarkable appearance of ephrinB2$^+$ ECs was clearly observed by the stimulation of cAMP pathway. That is, addition of AM induced ephrinB2$^+$ EC appearance (Figure 2B). AM with IBMX, or 8bromo-cAMP together with VEGF, showed substantial induction of ephrinB2$^+$ ECs (Figure 2C and 2D). Messenger RNA expression of arterial EC markers, ephrinB2, Dll4, Notch1, Notch4, Alk1, and neuropilin1 (NRP1) were increased in 8bromo-cAMP and VEGF-treated ECs (Figure 2E). In contrast, venous EC markers, COUP-TFII transcription factor[28] and NRP2[29] mRNA were decreased by

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