ACS partly through anti-inflammatory actions in addition to its lipid-lowering effects.[15,16] A previous study [17] showed that atorvastatin decreased the free plasma levels of VEGF in patients with CAD in the absence of AMI, but the study did not examine sFlt-1 levels and the possible clinical significance of the decrease in VEGF levels. Thus, we hypothesized that statin treatment may affect serial changes in the plasma levels of sFlt-1, VEGF, and PlGF over the time course of AMI, leading to an improvement of the depressed cardiac function that occurs after AMI. In this study, we determined the effect of atorvastatin on plasma levels of sFlt-1, VEGF, and PlGF over 6 months in patients with AMI, and examined the relation between changes in the plasma levels of these factors with post-MI left ventricular function.

Methods

Study Patients

This study included 50 consecutive patients with a first AMI who were admitted within 24 hours after the onset of symptoms and had successful reperfusion therapy with percutaneous coronary intervention immediately after admission to Yamanashi University Hospital. The diagnosis of AMI was made on the basis of chest pain persisting for ≥ 30 min, ST segment elevation of > 0.2 mV in ≥ 2 contiguous leads on a standard 12-lead electrocardiogram, and elevation of serum creatine kinase levels to more than twice the upper limit of normal. This study excluded patients with total cholesterol levels > 240 mg/dL or use of lipid-lowering medications at admission. This study also included 20 control subjects with angiographically normal coronary arteries and a normal left ventriculogram. The control subjects were selected to match the AMI patients for atherosclerosis risk factors, and plasma levels of sFlt-1, VEGF, and PlGF were compared between the AMI patients and controls subjects. The clinical characteristics of the patients with AMI and the control subjects are shown in Table 1. Written informed consent was obtained from all patients and

control subjects before the study. The study was in agreement with the guidelines approved by the ethics committee at our institution.

Study Protocol and Blood Sampling

The patients with AMI were randomly assigned to receive 6 months of oral atorvastatin (10 mg/day) or placebo (similar-appearing tablet) using a random number table generated by a computer. All patients were blinded to the content of the tablets. The treatment was started within 3 days after admission. All patients received instruction and counseling to promote compliance with lipid-lowering diet according to the National Cholesterol Education Program Adult Treatment Panel III guideline. Patients were not permitted to be treated with any lipid-modifying drugs other than the study drug.

At the acute phase of AMI, blood samples were obtained from an antecubital vein without stasis in all patients immediately after admission and before the administration of heparin. In 32 patients with AMI admitted within 6 hours after symptom onset, blood samples were taken every 4 hours over the first 24 hours for determination of creatine kinase levels. Furthermore, blood samples were also taken at 2 weeks and 6 months after AMI in the same manner from the antecubital vein without stasis in all patients with AMI. In the control subjects, blood samples were obtained from the antecubital vein without stasis. The blood samples, anticoagulated with EDTA, were immediately centrifuged at 3,000 rpm for 10 min at 4°C, and an aliquot of the EDTA-plasma was stored at -80°C until analyzed. Serum from a peripheral vein was also obtained at the same time.

Assays

Plasma levels of sFlt-1, VEGF, and PlGF were measured by ELISA assays (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. The sFlt-1 ELISA system is capable of detecting the total amount

of plasma sFlt-1 including that bound to VEGF and PlGF; the ELISA systems for VEGF and PlGF detect only free plasma levels of VEGF and PlGF not bound to sFlt-1. The minimal detection limits for sFlt-1, VEGF, and PlGF were 14.4 pg/mL, 9 pg/mL, and 7 pg/mL, respectively. C-reactive protein levels in the serum were assayed by rate nephelometry (Dade Behring, Marburg, Germany).

Cardiac Catheterization

Cardiac catheterization was performed immediately after admission at the acute phase of AMI, and at 2 weeks and 6 months after AMI in all patients with AMI. Left ventriculography was performed at 2 weeks and 6 months after AMI. Left ventricular ejection fraction (LVEF), LV end-diastolic volume index (LVEDVI), and LV end-systolic volume index (LVESVI) were determined with left ventriculograms by area-length methods using computer-assisted analysis (Cardio 2000, Fukuda-denshi Corporation, Tokyo, Japan).

Statistical Analysis

Data are expressed as mean ± SEM unless otherwise indicated. The mean value and frequency between 2 groups were compared using Student's unpaired *t* test and the Chi-square test, respectively. Chi-square test followed by Tukey test was used for comparing frequencies among the 3 patient groups. One-way analysis of variance followed by a Sheffe test for post-hoc comparisons was employed for comparisons of mean values before and during the treatment and of mean values among the 3 patient groups. The mean values of lipid and other biochemical parameters and cardiac function before and during treatment in the two AMI groups were compared using two-way analysis of variance for repeated measures followed by post-hoc testing with a Sheffe test. The relationship of sFlt-1, VEGF, and PlGF, ventricular function, and other parameters was examined by linear regression analysis. Statistical

significance was defined as $P < 0.05$. Analyses were performed in part using StatView 5.0 for Windows (Tokyo, Japan).

Results

Comparisons of baseline clinical characteristics

The study patients with AMI were randomly assigned to atorvastatin treatment in 25 patients and placebo in 25 patients. All of the study patients completed the trial. The clinical characteristics of the patients with AMI and the control subjects are shown in Table 1. Risk factor profiles were similar among the 2 treatment groups and the control subjects, as shown in Table 1. Clinical parameters associated with AMI were comparable between the 2 treatment groups with AMI, as shown in Table 1.

In the acute phase of MI (within 24 hours after AMI), sFlt-1 plasma levels were lower in patients with AMI than control subjects, while free VEGF and PlGF levels were higher in patients with AMI than control subjects, as shown in Fig 1. The placebo- and atorvastatin-treated groups with AMI had comparable levels of sFlt-1, VEGF, and PlGF in the acute phase of MI, as shown in Fig. 1.

Effects of treatments with atorvastatin and placebo during the follow-up period in patients with AMI

The effects of the treatments on lipid profiles, cardiac function, and other clinical features are shown in Table 2. The levels of total cholesterol and low density lipoprotein (LDL)-cholesterol were significantly lower in the atorvastatin group than the placebo group at 6 months after AMI, but the levels were comparable between the 2 treatment groups at 2 weeks after AMI. The frequencies of each of the cardiac medications were similar except for atorvastatin between the 2 treatment groups during the follow-up period.

LVEF in the atorvastatin group was comparable at 2 weeks after AMI

but significantly greater at 6 months after AMI compared with the placebo group, as shown in Fig. 2 and Table 2. LVEDVI and LVESVI in the atorvastatin group showed a tendency to be lower at 6 months after AMI than the placebo group, but it did not reach significance, as shown in Table 2.

Plasma levels of sFlt-1, VEGF, and PlGF were not significantly different among the 2 treatment groups at 2 weeks after AMI and the control subjects, as shown in Fig. 1. The plasma levels of sFlt-1, VEGF, and PlGF were unchanged from 2 weeks to 6 months after AMI in the placebo group. However, in the atorvastatin group, sFlt-1 levels increased from 2 weeks to 6 months after AMI, while VEGF and PlGF levels reciprocally decreased from 2 weeks to 6 months after AMI, as shown in Fig. 1.

Correlations of changes in levels of sFlt-1, VEGF, or PlGF and ventricular function during treatment in patients with AMI

The percent changes in LVEF were correlated positively with the percent changes in sFlt-1 levels and inversely with the percent changes in VEGF and PlGF levels from 2 weeks to 6 months after AMI, as shown in Fig. 3.

The percent changes in LDL-cholesterol and C-reactive protein levels did not show a significant correlation with percent changes in sFlt-1, VEGF, and PlGF levels and in LVEF from 2 weeks to 6 months after AMI (LDL-cholesterol levels, r = - 0.1, 0.1, 0.05, and - 0.14, respectively; C-reactive protein levels, r = 0.15, - 0.19, 0.2, and 0.05, respectively).

Discussion

The present study showed that sFlt-1 levels were elevated and VEGF and PlGF levels were reciprocally decreased at the acute phase of MI as compared with healthy control subjects. Furthermore, by 2 weeks after MI in both the atorvastatin and placebo groups, these levels returned to levels similar

to those obtained in healthy control subjects. The elevation of VEGF and PlGF levels at the acute phase of MI may be an adaptive mechanism for inducing neo-vascularization in ischemic myocardium,[1-3,18,19] and may be caused in part through a mechanism involving hypoxia-inducible factor-1 activation.[20] The decreased levels of sFlt-1, a natural antagonist for VEGF and PlGF, may be helpful for the angiogenic activity of VEGF and PlGF in ischemic myocardium in the acute phase of MI. Furthermore, the present study showed that atorvastatin treatment induced an increase in sFlt-1 levels and a reciprocal decrease in VEGF and PlGF levels at 6 months after MI compared to placebo treatment. Moreover, these changes in plasma levels were correlated with improvement of post-MI ventricular dysfunction from 2 weeks to 6 months after AMI. Change in LDL-cholesterol levels was not related to the improvement of LV function and changes in sFlt-1, VEGF, and PlGF levels from 2 weeks to 6 months after AMI. Therefore, the increase in sFlt-1 levels and the reciprocal decrease in VEGF and PlGF levels after atorvastatin treatment may have clinical benefit in chronic phase of MI in patients with AMI independently of decrease in lipids levels. However, the effect of atorvastatin-induced changes in plasma levels of sFlt-1, VEGF, and PlGF on long-term clinical outcome remains to be determined.

VEGF and PlGF belong to the same gene family and play a synergistic role in pathologic angiogenesis.[1-3,19] VEGF exerts its biological activities by interacting with 2 receptors, Flt-1 and Flk-1/KDR, while PlGF interacts with Flt-1 but not Flk-1/KDR.[1-4,6,7,19] It has been shown that Flk-1/KDR, expressed on vascular endothelium, mainly mediates VEGF-induced angiogenesis.[1-3,19] On the other hand, Flt-1 is expressed on endothelial cells and inflammatory cells, namely monocytes and macrophages.[1-3,19] Thus, VEGF and PlGF also have pro-inflammatory properties and thereby both angiogenic growth factors have the capability of promoting atherosclerosis through Flt-1. This is supported by previous reports[13,14] showing that elevated levels of VEGF and

PlGF in ACS resulted in an adverse prognosis. However, it remains unclear whether elevated VEGF and PlGF levels in ACS may function as a pro-atherogenic factor via Flt-1 or may only serve as a surrogate marker of myocardial injury. sFlt-1, generated by alternative splicing of the Flt-1 gene, reduces effective plasma concentrations of VEGF and PlGF and inactivates their activities.[4-10] Furthermore, sFlt-1 forms heterodimers with membrane-bound Flt-1[4] and thus acts as a receptor blocker of Flt-1. Thus, sFlt-1 could potentially attenuate the adverse effects of VEGF and PlGF and may have a beneficial effect in ACS. In fact, a previous report [21] demonstrated that the increase in sFlt-1 plasma levels by its gene transfer inhibited vascular inflammation and prevented subsequent atherosclerosis in an animal model. On the other hand, previous reports [5,22] using an animal model showed that exogenous administration of sFlt-1 acutely induced hypertension and nephro-toxicity probably due to inhibition of the endothelial protective action of endogenous VEGF. However, the sFlt-1 plasma levels exogenously administered were nearly ten- or 100-times higher than the physiological plasma levels in these animal experiments.[5,22] Furthermore, coronary artery disease is a long-term process in humans. Therefore, the role of elevated levels of endogenous sFlt-1 in human atherosclerotic cardiovascular disease still remains largely unknown.

sFlt-1 has been shown to be expressed in vascular endothelium as well as placenta, and alternative splicing has been identified as a key regulatory step in sFlt-1 production.[1-4] However, the regulatory mechanisms by which sFlt-1 levels decreased during the acute phase of MI in the present study and thereafter increased in response to atorvastatin treatment remain unknown.

In conclusion, there was a reciprocal relationship between changes in sFlt-1 levels and VEGF and PlGF levels after MI; atorvastatin increased sFlt-1 levels while decreasing VEGF and PlGF levels. These changes were associated with late improvement of ventricular function after AMI, and may represent an

additional benefit of statin therapy.

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Figure Legends

Fig 1. Comparison of sFlt-1, VEGF, and PlGF levels during the follow-up period after AMI in patients treated with atorvastatin (n = 25) and placebo (n = 25). *$P < 0.05$ vs. respective levels in the acute phase of MI. †$P < 0.05$ vs. healthy control subjects.

Fig 2. Comparison of changes in left ventricular ejection fraction (LVEF) from 2 weeks to 6 months after AMI in patients treated with atorvastatin (n = 25) and placebo (n = 25).

Fig 3. Correlations between percent changes in LVEF and in plasma levels of sFlt-1, VEGF, or PlGF from 2 weeks to 6 months after AMI in patients treated with atorvastatin (n = 25, closed circles) and placebo (n = 25, open circles).

Table 1. Patients' characteristics at baseline

	Acute myocardial infarction		Controls	p value
	Atorvastatin (n = 25)	Placebo (n = 25)	(n = 20)	
Age (yrs)	64.5 ± 2.1	62.7 ± 2.2	64.1 ± 1.1	n.s.
Male (%)	68	72	75	n.s.
BMI (kg/m2)	23.2 ± 0.6	24.5 ± 0.6	24.5 ± 0.6	n.s.
Smoking (%)	56	48	55	n.s.
Hypertension (%)	72	64	70	n.s.
Diabetes mellitus (%)	60	64	55	n.s.
Total cholesterol (mg/dl)	196 ± 8	202 ± 8	198 ± 5	n.s.
Triglyceride (mg/dl)	133 ± 17	143 ± 12	124 ± 8	n.s.
HDL cholesterol (mg/dl)	46 ± 3	45 ± 3	49 ± 2	n.s.
LDL cholesterol (mg/dl)	120 ± 6	116 ± 7	115 ± 4	n.s.
Location of MI (%)				
Anterior	52	56	-	n.s.
Inferior	28	20	-	n.s.
Others	20	24	-	n.s.
Extent of CAD (%)				
1-vessel	36	48	-	n.s.
2-vessel	40	32	-	n.s.
3-vessel	24	20	-	n.s.
Killip classification (%)				
class I	72	64	-	n.s.
class II~IV	28	36	-	n.s.
peak CKMB (ng/ml)	266 ± 45	294 ± 77	-	n.s.
Time to reperfusion (hrs)	6.4 ± 0.9	6.1 ± 0.8	-	n.s.

Values represent % of the patients and control subjects or mean ± SE. BMI, body mass index; HDL, high density lipoprotein; LDL, low density lipoprotein; MI, myocardial infarction; CAD, coronary artery disease; CK, creatine kinase; CKMB, creatine kinase MB. Smoking, defined as smoking ≥10 cigarettes/day for ≥10 years; Hypertension, defined as >140/90 mm Hg or use of antihypertensive medication; Diabetes mellitus, defined according to the American Diabetes Association report or as taking an antidiabetic medication.

Table 2. Risk factors, cardiac functions, and medications during the follow up period in patients with acute myocardial infarction

	Atorvastatin (n = 25)		Placebo (n = 25)	
	2 weeks	6 months	2 weeks	6 months
Risk factors				
Total cholesterol (mg/ml)	201 ± 8	166 ± 8*†	209 ± 8	201 ± 8
LDL cholesterol (mg/ml)	130 ± 6	87 ± 8*†	122 ± 7	116 ± 7
HDL cholesterol (mg/ml)	49 ± 3	54 ± 3	46 ± 3	50 ± 3
Triglyceride (mg/ml)	134 ± 15	117 ± 13	139 ± 12	133 ± 21
HbA1c (%)	6.2 ± 0.3	6.0 ± 0.2	6.2 ± 0.2	6.0 ± 0.2
Systolic BP (mmHg)	129 ± 6	124 ± 5	123 ± 4	126 ± 4
CRP (mg/ml)	0.80 ± 0.26	0.11 ± 0.05*	0.74 ± 0.20	0.16 ± 0.03*
BMI (kg/m^2)	22.8 ± 0.5	23.0 ± 0.6	23.5 ± 0.5	24.4 ± 0.6
Cardiac Function				
LVEF (%)	54.3 ± 2.3	63.5 ± 2.7*†	53.5 ± 2.0	55.2 ± 2.6
LVEDVI (ml/m^2)	94.2 ± 3.3	94.0 ± 3.1	95.8 ± 4.9	99.1 ± 3.5
LVESVI (ml/m^2)	41.2 ± 4.4	34.0 ± 4.3	42.3 ± 4.7	43.5 ± 6.3
LVEDP (mmHg)	13.1 ± 0.9	13.8 ± 0.9	14.3 ± 1.5	14.0 ± 0.7
CI (l/min/m^2)	2.9 ± 0.1	3.0 ± 0.1	2.8 ± 0.7	2.8 ± 0.1
Medications				
ACE-I (%)	76	64	64	52
ARB (%)	24	32	36	40
β-blocker (%)	20	16	12	8
Ca-blocker (%)	36	44	32	40
Aspirin (%)	100	96	100	100
Ticlopidine (%)	76	20*	72	20*
SU (%)	8	8	8	12
Insulin (%)	12	12	8	8

Values represent % of the patients and mean ± SE. *$p < 0.05$ vs. 2 weeks, †$p < 0.05$ vs. placebo. BP, blood pressure; LVEF, left ventricular ejection fraction; LVEDVI, left ventricular end diastolic volume index; LVESVI, left ventricular end systolic volume index; LVEDP, left ventricular end diastolic pressure; CI, cardiac index; ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensis II receptor blocker, SU; sulfonylureas. Other abbreviations are as Table 1.

Fig 1

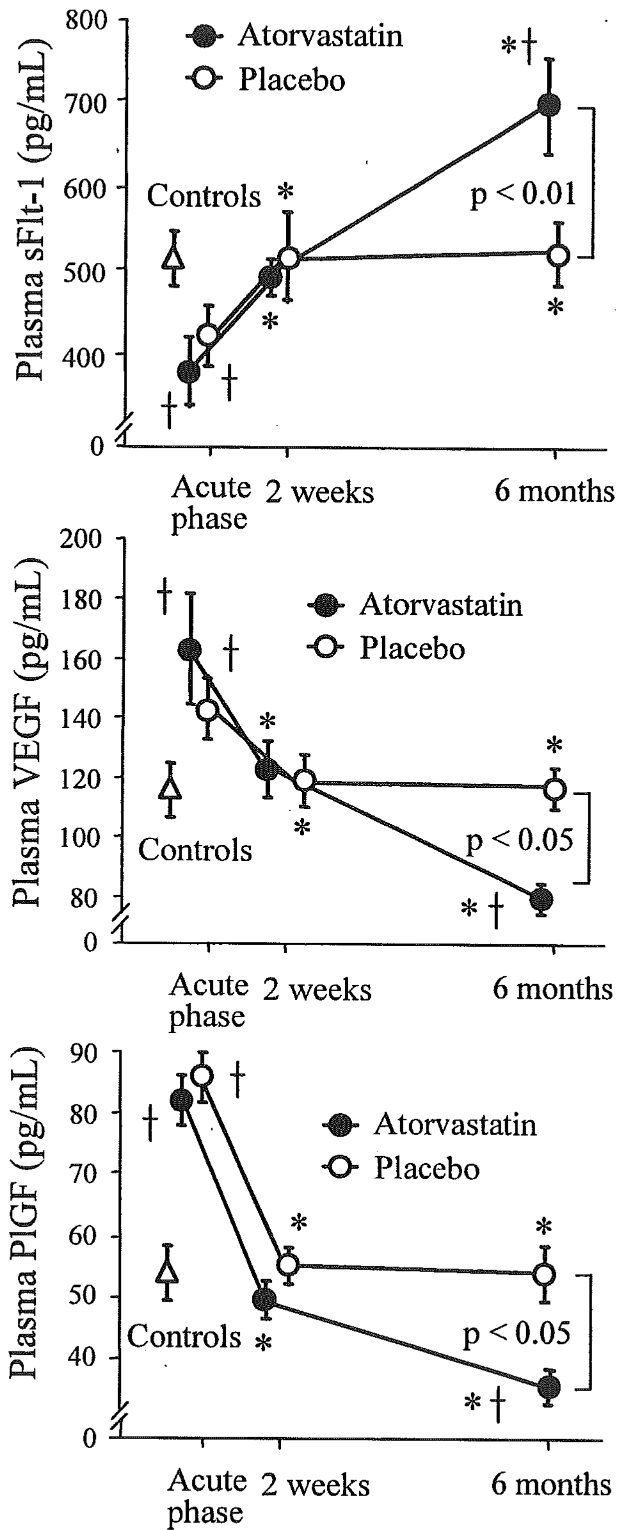
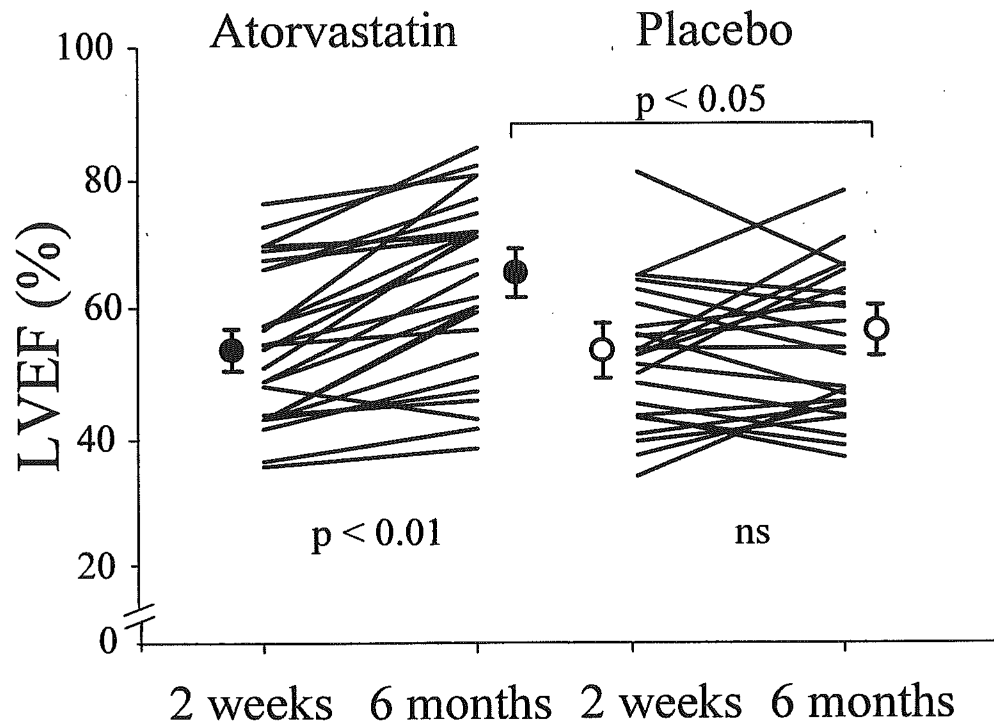


Fig 2



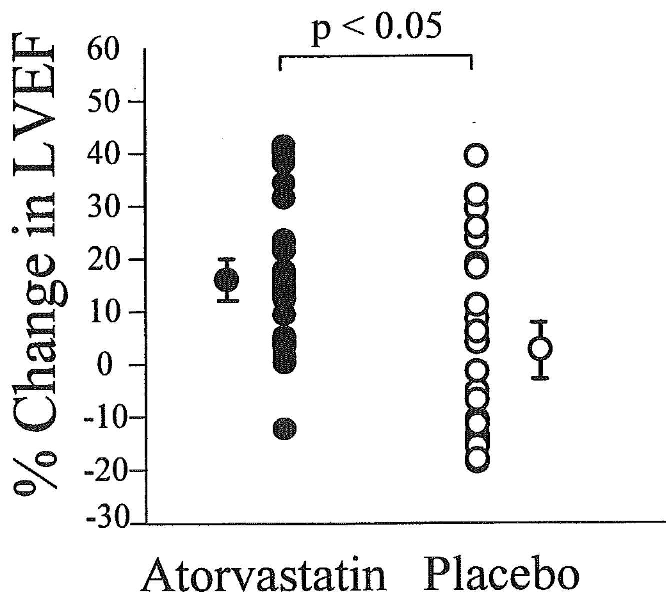
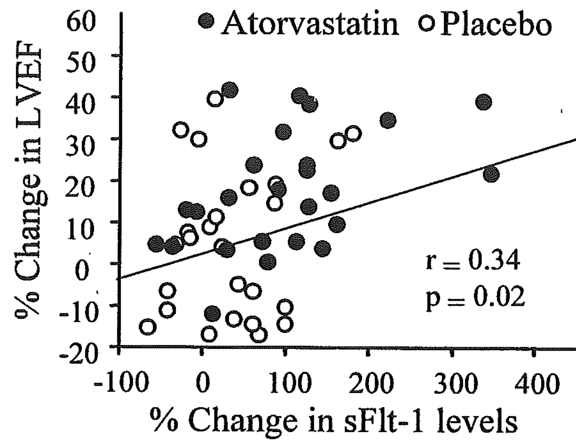
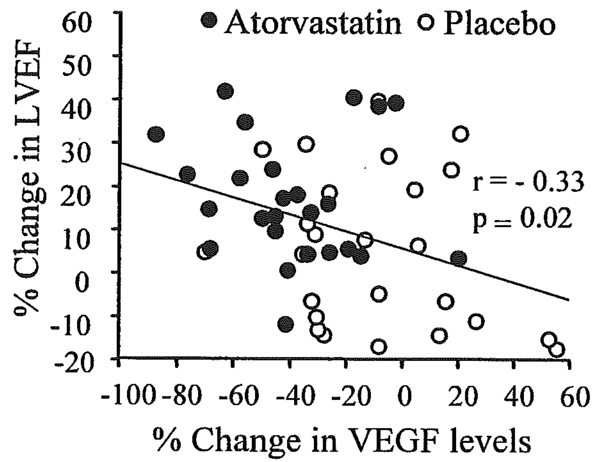
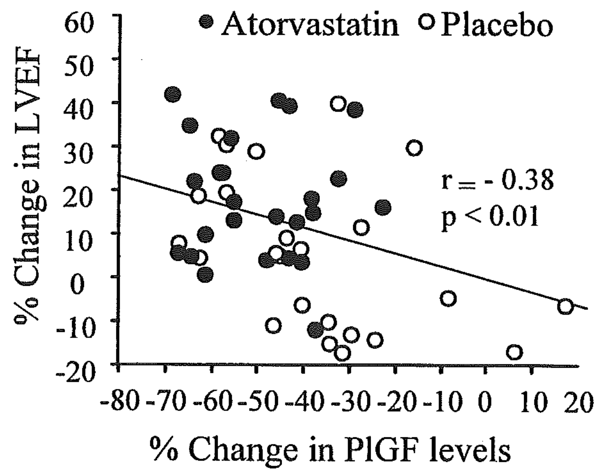


Fig 3







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Role of adiponectin receptors in endothelin-induced cellular hypertrophy AQ: 1 in cultured cardiomyocytes and their expression in infarcted heart

Daisuke Fujioka, Ken-ichi Kawabata, Yukio Saito, Tsuyoshi Kobayashi, Takamitsu Nakamura, Yasushi Kodama, Hajime Takano, Jyun-ei Obata, Yoshinobu Kitta, Ken Umetani, and Kiyotaka Kugiyama

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Fujioka, Daisuke, Ken-ichi Kawabata, Yukio Saito, Tsuyoshi Kobayashi, Takamitsu Nakamura, Yasushi Kodama, Hajime Takano, Jyun-ei Obata, Yoshinobu Kitta, Ken Umetani, and Kiyotaka Kugiyama. Role of adiponectin receptors in endothelin-induced cellular hypertrophy in cultured cardiomyocytes and their expression in infarcted heart. *Am J Physiol Heart Circ Physiol* 290: H000–H000, 2006. First published January 13, 2006; doi:10.1152/ajpheart.00987.2005.—Adiponectin, an adipocyte-derived protein, has cardioprotective actions. We elucidated the role of the adiponectin receptors AdipoR1 and AdipoR2 in the effects of adiponectin on endothelin-1 (ET-1)-induced hypertrophy in cultured cardiomyocytes, and we examined the expression of adiponectin receptors in normal and infarcted mouse hearts. Recombinant full-length adiponectin suppressed the ET-1-induced increase in cell surface area and [^{3}H]leucine incorporation into cultured cardiomyocytes compared with cells treated with ET-1 alone. Transfection of small interfering RNA (siRNA) specific for AdipoR1 or AdipoR2 reversed the suppressive effects of adiponectin on ET-1-induced cellular hypertrophy in cultured cardiomyocytes. Adiponectin induced phosphorylation of AMP-activated protein kinase (AMPK) and inhibited ET-1-induced ERK1/2 phosphorylation, which were also reversible by transfection of siRNA for AdipoR1 or AdipoR2 in cultured cardiomyocytes. Transfection of siRNA for α_2-catalytic subunits of AMPK reduced the inhibitory effects of adiponectin on ET-1-induced cellular hypertrophy and ERK1/2 phosphorylation. Effects of globular adiponectin were similar to those of full-length adiponectin, and siRNA for AdipoR1 reversed the actions of globular adiponectin. Compared with normal left ventricle, expression levels of AdipoR1 mRNA and protein were decreased in the remote, as well as the infarcted, area after myocardial infarction in mouse hearts. In conclusion, AdipoR1 and AdipoR2 mediate the suppressive effects of full-length and globular adiponectin on ET-1-induced hypertrophy in cultured cardiomyocytes, and AMPK is involved in signal transduction through these receptors. AdipoR1 and AdipoR2 might play a role in the pathogenesis of ET-1-related cardiomyocyte hypertrophy after myocardial infarction.

AMP-activated protein kinase; small interfering RNA; myocardial infarction

AQ: 2 ADIPONECTIN, AN ABUNDANT CIRCULATING protein secreted from adipose tissue, plays a fundamental role in energy homeostasis and glucose and lipid metabolism in adipose tissue and has insulin-sensitizing effects on liver and skeletal muscle (3, 5, 9, 12). Shibata et al. (15, 16) recently demonstrated that adiponectin suppresses cardiac hypertrophy in response to pressure overload and protects the heart from ischemia-reperfusion injury. Recently, it has been shown that AMP-activated protein kinase (AMPK), an important regulator of the adiponectin signaling pathway (22), not only improves myocardial glucose and lipid metabolism but also prevents ventricular contractile dysfunction in the ischemic heart (14). It is also known that abnormalities in glucose and lipid metabolism in cardiac muscle are associated with heart failure (6, 13). Thus it is possible that adiponectin might exert cardioprotective properties in various heart diseases. Adiponectin exerts its action through two recently discovered receptors, AdipoR1 and AdipoR2 (21). Previous reports (2, 17) have shown that skeletal muscle produces adiponectin and expresses adiponectin receptors. However, the expression remains unclarified in cardiac muscle. Cardiac hypertrophy in the remote area of the infarcted heart is initially a compensatory response of myocardial tissue to increased mechanical load, but its early beneficial effects become maladaptive, leading to heart failure at a later phase of myocardial infarction (8, 19, 23). Among several neurohumoral factors activated after myocardial infarction, endothelin-1 (ET-1) plays an important role in the genesis of myocyte hypertrophy after myocardial infarction (8, 23). Thus this study examined the possible role of AdipoR1 and AdipoR2 in ET-1-induced cellular hypertrophy in cultured cardiomyocytes and AdipoR1 and AdipoR2 expression in infarcted hearts in animal models. The results demonstrate a potential role for the cardiac adiponectin system in the pathogenesis of cardiac hypertrophy.

MATERIALS AND METHODS

Materials. Rat recombinant full-length adiponectin was purchased from BioVision (Mountain View, CA) and globular adiponectin from Adipogen (Sungnam, Korea). Both adiponectins were derived from bacteria (*Escherichia coli*). The full-length adiponectin forms monomers, trimers, hexamers, and high-molecular-weight multimers, and the globular adiponectin forms monomers, dimers, and trimers. Anti-AdipoR1 and anti-AdipoR2 polyclonal antibodies were purchased from Alpha Diagnostic International

AQ: 7 Address for reprint requests and other correspondence: K. Kugiyama, Dept. of Internal Medicine II, Interdisciplinary Graduate School of Medicine and Engineering, Univ. of Yamanashi, 1110 Shimokato, Nakakoma-gun, Yamanashi 409-3898, Japan (e-mail: kugiyama@yamanashi.ac.jp).

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(San Antonio, TX). Anti-adiponectin and anti-ERK polyclonal antibodies were obtained from R & D Systems (Minneapolis, MN). Anti-phosphorylated AMPK (Thr^{172}), anti-pan-α-AMPK, anti-phosphorylated acetyl CoA-carboxylase (ACC), and anti-ACC polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA). Anti-phosphorylated ERK (Thr^{202}/Tyr^{204}) and anti-β-tubulin polyclonal antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture reagents were purchased from Sigma (Tokyo, Japan) and Invitrogen (Carlsbad, CA). ET-1, TNF-α, insulin-like growth factor-I (IGF-I), 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR), and other chemicals were purchased from Sigma.

Preparation and culture of rat cardiomyocytes. Primary cultures of rat neonatal cardiomyocytes were prepared by trypsin-EDTA digestion from ventricles of 1- to 3-day-old Sprague-Dawley rats as described previously (15). Briefly, after trypsinization, the cells were collected by ultracentrifugation and diluted to 5×10^6 cells/ml in DMEM containing 10% FCS. The cells were preplated and cultured for 30 min to eliminate nonmyocardial cells. Nonattached cells were suspended in DMEM containing 10% FCS and plated for 72 h on plastic petri dishes. After the cells were washed, the medium was replaced with DMEM containing 0.5% FCS for 12 h before each experiment.

Measurements of mRNA and protein expression levels in myocardium and cultured cardiomyocytes. Total RNA was extracted from myocardial tissues, skeletal muscle (soleus muscle), intraperitoneal adipose tissue of rats and mice, and rat cultured cardiomyocytes with the RNeasy kit and DNase I (Qiagen). Expression levels of mRNA for adiponectin, AdipoR1, and AdipoR2 were quantified by a real-time two-step RT-PCR assay with use of SYBR green chemistry, based on the 5′-nuclease activity of *Taq* polymerase, and a sequence detection system (GeneAmp 5700, PE Applied Biosystems, Foster City, CA). The PCR primers are listed in Table 1. The GAPDH housekeeping gene was used for normalization of target gene expression.

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Table 1. *Sequences of sense siRNAs and PCR primers*

Primers	Sequences
	Real-time PCR
Mouse adiponectin	
Forward	5′-GCAAGCTCTCCTGTTCCTCTTAATG-3′
Reverse	5′-TGCATCTCCTTTCTCTCCCTTCTC-3′
Mouse AdipoR1	
Forward	5′-ACGTTGGAGAGTCATCCCGTAT-3′
Reverse	5′-CTCTGTGTGGATGCGGAAGAT-3′
Mouse AdipoR2	
Forward	5′-TCCCAGGAAGATGAAGGGTTTAT-3′
Reverse	5′-TTCCATTCGTTCCATAGCATGA-3′
Rat AdipoR1	
Forward	5′-TCTTCCTCATGGCTGTGATG-3′
Reverse	5′-AGCACTTGGGAAGTTCCTCC-3′
Rat AdipoR2	
Forward	5′-GGAGCCATTCTCTGCCTTTC-3′
Reverse	5′-ACCAGATGTCACATTTGCCA-3′
	siRNA
Rat AdipoR1 siRNA	UACAACACCACUCAAGCCAAGUCCC
Rat AdipoR2 siRNA	AACAGGUGUCUCUAAACUGGGCUCC
Rat AMPKα$_2$ siRNA	AUAAGCCACUGCGAGCUGGUCUUGA
Rat unrelated siRNA	AUUUAACUUCUGGUGACGAUACUGG

siRNA, small interfering RNA; AMPK, AMP-activated protein kinase; AdipoR1 and AdipoR2, adiponectin receptors.

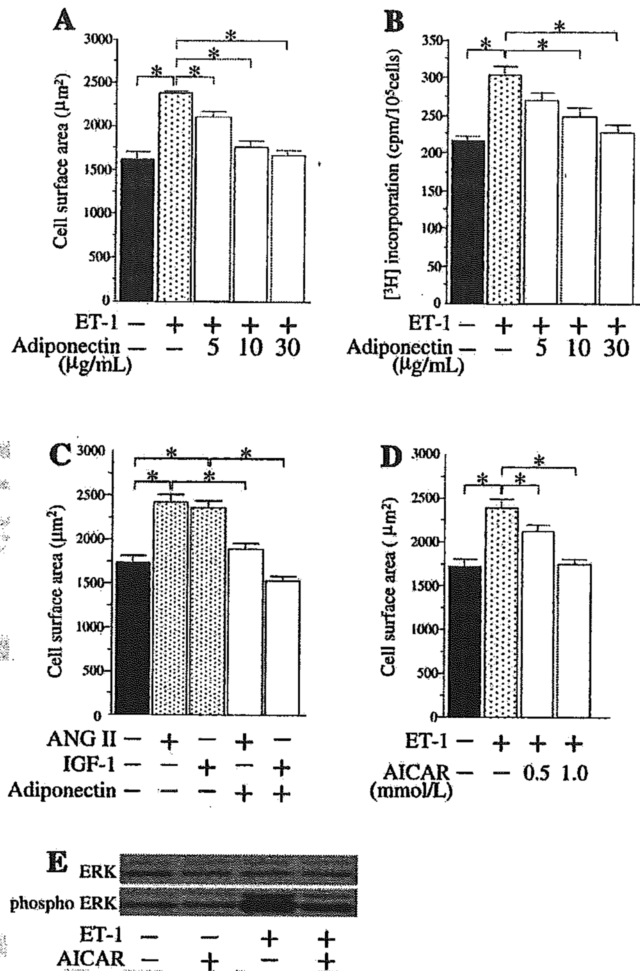

Fig. 1. Effects of adiponectin or 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside (AICAR) on hypertrophic responses to endothelin-1 (ET-1), ANG II, and insulin-like growth factor-I (IGF-I) and ET-1-induced ERK phosphorylation in cultured neonatal rat cardiomyocytes. Cultured cardiomyocytes were treated with or without full-length adiponectin or AICAR (1 mmol/l) and then incubated with ET-1 (100 nmol/l), ANG II, or IGF-I. Values are means ± SE (n = 6). *P < 0.05. *A*: effect of adiponectin on cell surface area in response to ET-1. *B*: effect of adiponectin on protein synthesis evaluated by [^{3}H]leucine incorporation [counts per minute (cpm) per 10^5 cells] in response to ET-1. *C*: effects of adiponectin (30 μg/ml) on cell surface area in response to ANG II (100 nmol/l) or IGF-I (100 nmol/l). *D*: effect of AICAR on cell surface area in response to ET-1. *E*: effect of AICAR on ET-1-induced ERK1/2 phosphorylation.

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For immunoblot analysis, the extracts of myocardial tissue, skeletal muscle, and intraperitoneal adipose tissue of rats and mice or the treated cells were matched for protein concentration (15 μg) with SDS-PAGE sample buffer and separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. The membranes were incubated with the indicated primary antibodies overnight at 4°C and then with horseradish peroxidase-conjugated secondary antibody at a 1:20,000 dilution. The ECL Plus Western Blotting Detection System (Amersham Biosciences, Piscataway, NJ) was used for detection. Intensity of the β-tubulin band was used as a loading control between samples.

Measurement of protein synthesis and cell surface area in cultured cardiomyocytes. Protein synthesis in cultured cardiomyocytes was evaluated by incorporation of [^{3}H]leucine into the cells as described in

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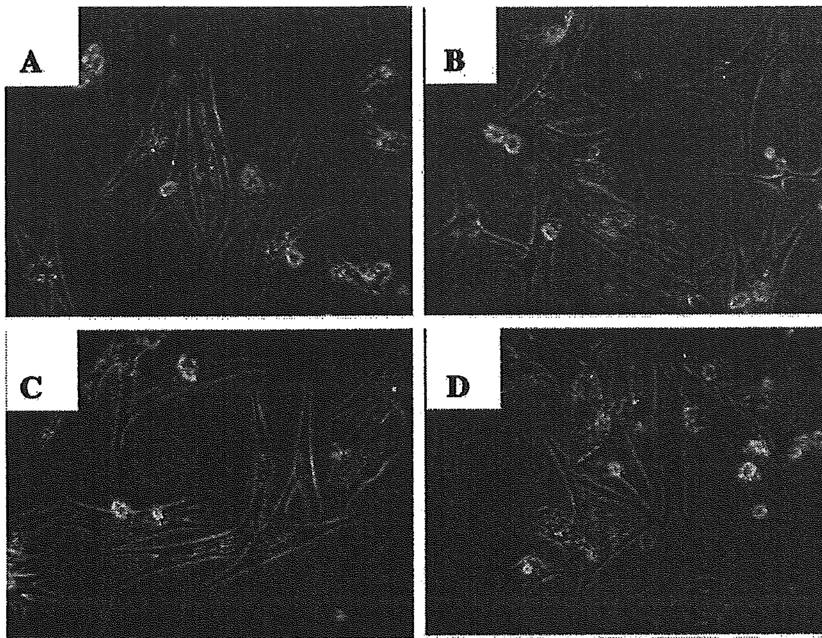

Fig. 2. Photomicrographs of cultured cardiomyocytes treated with PBS (*A*), ET-1 (100 nmol/l; *B*), full-length adiponectin (30 μg/ml) + ET-1 (100 nmol/l; *C*), or combination of small interfering RNAs (siRNAs) with AdipoR1 and AdipoR2 and adiponectin (30 μg/ml) + ET-1 (100 nmol/l; *D*).

previous reports (11, 15). Briefly, cardiomyocytes on a 24-well plate were pretreated with or without adiponectin for 4 h. The cells were incubated for 42 h with or without ET-1 (100 nmol/l) and for an additional 6 h with 1 μCi/ml [^{3}H]leucine (Amersham). The cultures were washed twice with ice-cold PBS and fixed with 10% TCA (Sigma). After the cultures were washed, radioactivity in the TCA-precipitable materials was determined after solubilization in 0.25 N NaOH.

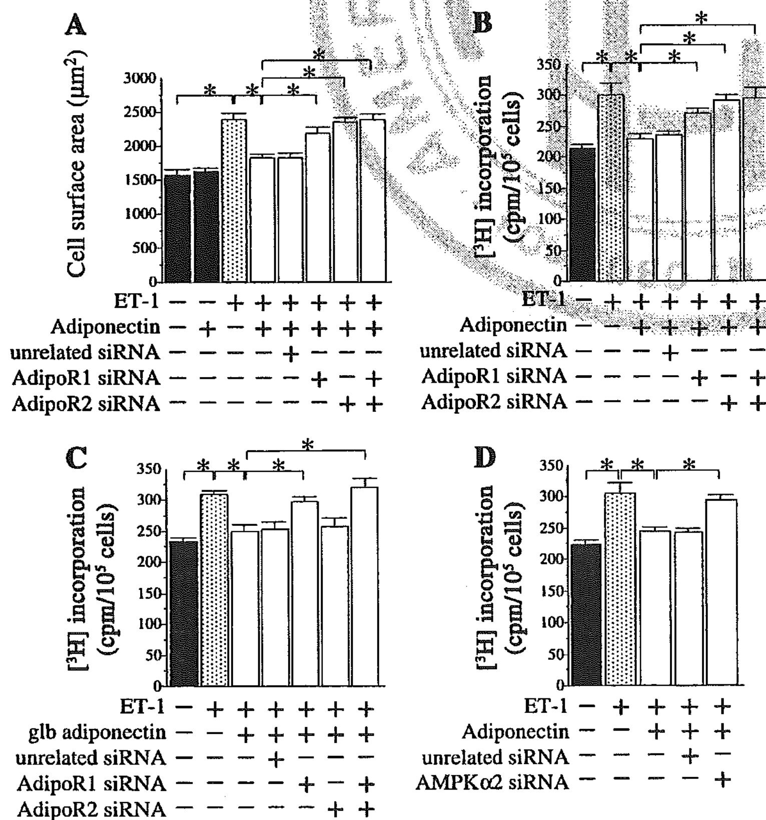

Fig. 3. Effects of suppression of AdipoR1, AdipoR2, or AMP-activated protein kinase (AMPK)-α_2 by siRNAs on inhibitory action of adiponectin on ET-1-induced hypertrophic responses in cultured cardiomyocytes. After transfection of small interfering RNA (siRNA), cardiomyocytes were pretreated with full-length (30 μg/ml) or globular (2.5 μg/ml) adiponectin and then incubated with ET-1 (100 nmol/l), and cell surface area and [^{3}H]leucine incorporation were measured. Values are means ± SE ($n = 6$). *$P < 0.05$. *A*: effects of AdipoR1 siRNA, AdipoR2 siRNA, combining siRNAs, or unrelated siRNA on inhibitory action of full-length adiponectin on ET-1-induced increase in surface area. *B*: effects of siRNAs on inhibitory action of full-length adiponectin on ET-1-induced increase in [^{3}H]leucine incorporation. *C*: effects of siRNAs on inhibitory action of globular (glb) adiponectin on ET-1-induced increase in [^{3}H]leucine incorporation. *D*: effect of AMPKα_2 siRNA on inhibitory action of full-length adiponectin on ET-1-induced increase in [^{3}H]leucine incorporation.