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Impact of Metabolic Syndrome on the Long-Term Survival of Patients With Acute Myocardial Infarction

—— Potential Association With C-Reactive Protein ——

Masayoshi Takeno, MD; Satoshi Yasuda, MD†*; Yoritaka Otsuka, MD*; Isao Morii, MD‡; Atsushi Kawamura, MD*; Katsusuke Yano, MD**; Shunichi Miyazaki, MD†

Background Population-based cohort studies demonstrate that metabolic syndrome (MeS) is associated with increased risk for cardiovascular diseases and related mortalities. The present study was designed to investigate the prognostic impact of MeS in patients with acute myocardial infarction (AMI).

Methods and Results The study group was 461 AMI patients without a history of previous myocardial infarction. On the basis of the National Cholesterol Education Program Adult Treatment Panel III criteria, MeS was defined having at least 3 of the following 5 conditions: dysglycemia (impaired fasting glucose, current use of insulin or oral hypoglycemic drugs), hypertriglyceridemia, low high-density lipoprotein-cholesterol level, hypertension and obesity. The prevalence of MeS was 37% (n=172). C-reactive protein (CRP) levels increased with the increase in the number of conditions of MeS. During follow-up at a median of 17.6 months, the incidence of major adverse cardiovascular events (MACE) was significantly different between patients with and without MeS. Furthermore, after adjustment of predictive factors (age, sex, Killip class, multivessel coronary artery disease, low ejection fraction and high CRP level), MeS was an independent risk factor for MACE.

Conclusions In patients with AMI, MeS is associated with systemic inflammation and is an important predictor for MACE, which suggests the need for early identification and medical intervention for secondary prevention of MeS. (*Circ J* 2008; **72:** 415–419)

Key Words: Glucose; Inflammation; Metabolic syndrome; Myocardial infarction

Several population-based studies have shown that metabolic syndrome (MeS) is an independent predictor of cardiovascular diseases, including acute myocardial infarction (AMI)[1–3] It has also become clear that MeS is strongly associated with systemic inflammation characterized by high levels of C-reactive protein (CRP)[4,5]

Although the number of deaths caused by AMI has declined over the past decade, the incidence of recurrent myocardial infarction (MI) is unchanged[6,7] which indicates the importance of understanding the underlying risk factors that lead to secondary cardiac events. Therefore, this study was designed to investigate the long-term prognostic impact of MeS in patients with AMI.

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*Department of Cardiovascular Medicine, Course of Medical and Dental Sciences, Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, *Division of Cardiology, National Cardiovascular Center, Suita, **Faculty of Health Management, Nagasaki International University, Sasebo, †Division of Cardiology, Department of Internal Medicine, Kinki University School of Medicine, Osakasayama, ‡‡Division of Cardiology, Department of Internal Medicine, Tohoku University School of Medicine, Sendai and ‡Division of Cardiology, Hokusetsu General Hospital, Takatsuki, Japan*

Mailing address: Shunichi Miyazaki, MD, Division of Cardiology, Department of Internal Medicine, Kinki University School of Medicine, 377-2 Ohno-Higashi, Osakasayama 589-8511, Japan. E-mail: smiyazak@med.kindai.ac.jp

Methods

Study Patients

From January 2000 to December 2002, 465 patients who had an AMI without a previous MI were admitted to the coronary care unit of the National Cardiovascular Center, Japan. Four patients complicated with severe inflammatory diseases such as sepsis, pneumonia and pyelonephritis were excluded, leaving a total of 461 patients who were retrospectively analyzed in the present study. The study protocol was approved by the institutional review board.

Definitions

AMI was defined as the presence of any 2 of the following 3 conditions: typical chest pain for at least 30 min, typical electrocardiogram changes (ST elevation, ST depression, T inversion and new pathological Q waves in at least 2 adjacent leads) and elevation of serum creatine kinase level to more than twice the upper normal limit.

Significant coronary artery stenosis was defined as stenosis in more than 75% of the vessels. Multivessel coronary disease was defined as a significant stenosis of 1 or more vessels other than the infarct-related artery. Left main coronary disease was considered to be double vessel involvement. Left ventricular ejection fraction (LVEF) was measured by the Simpson's method on left ventriculography or echocardiography, and left ventricular dysfunction was defined as a LVEF <40%. Congestive heart failure at admission was diagnosed on the basis of physical examination, such as presence of moist rales on chest auscultation and

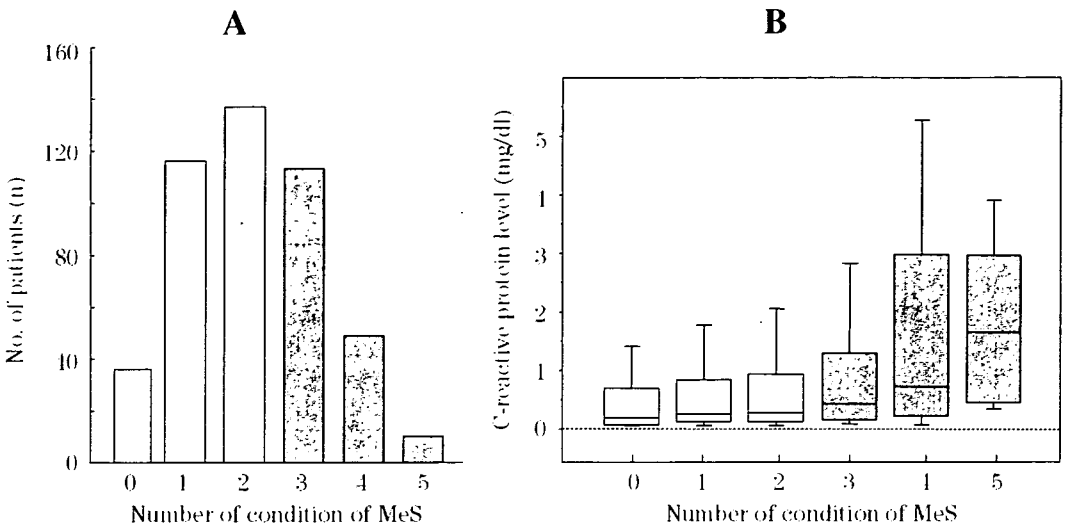

Fig 1. (A) Distribution of the number of conditions of metabolic syndrome (MeS) among 461 patients with acute myocardial infarction. (B) Distribution of C-reactive protein level according to the number of conditions of MeS. Box plots demonstrate median, and 25th, and 75th percentile values for C-reactive protein.

pulmonary congestion on the chest roentgenogram.

The presence of atherosclerotic disease was confirmed by a history of cerebral infarction, presence of arteriosclerosis obliterans or aortic aneurysm.

MeS was defined as having at least 3 of the following 5 conditions set by the recent National Cholesterol Education Program (NCEP) Adult Panel III (ATP-III) report[8] with modifications: dysglycemia, hypertension, hypertriglyceridemia, decreased level of high-density lipoprotein (HDL)-cholesterol, and obesity. Dysglycemia was defined as a fasting glucose >6.11 mmol/L; for the purpose of this analysis, the dysglycemia definition was also met by current use of insulin or oral hypoglycemic drugs. Hypertension was defined as a systolic blood pressure >130 mmHg and/or a diastolic blood pressure >85 mmHg; for the purpose of this analysis, hypertension was also met by current use of antihypertensive drugs. Hypertriglyceridemia was defined as a serum triglyceride level >1.69 mmol/L. Low HDL-cholesterol was defined as a serum level <1.03 mmol/L in men and <1.29 mmol/L in women. Obesity was defined as a body mass index >25 kg/m^2 according to the guidelines of the Japan Society for the Study of Obesity, because the body structure of Japanese is smaller than that of Caucasians and Africans and therefore the World Health Organization criteria appear to be inappropriate for Japanese[9,10]

In addition, the CRP level was defined as high if >0.3 mg/dl, on the basis of previous studies[4,11,12]

Laboratory Measurements

To assess the glycemic and lipid profiles, venous blood samples were drawn in the morning during fasting conditions in the stable phase of AMI (median: 9 days from the onset of AMI). Serum glucose concentration was measured using a glucose oxidase method (Glucose GA-1140; Arkray, Kyoto, Japan). Total cholesterol, triglyceride and HDL-cholesterol concentrations were determined by enzymatic methods using a Toshiba TBA 80M analyzer (Toshiba, Japan). Low-density lipoprotein was calculated using Friedewald's formula.

Serum CRP level was measured by the N Latex CRP II monoassay using a nephelometric technique with a nephelometric analyzer (BN II, Dade Behring, Germany). Additional measurements of CRP level were also performed in the acute phase (median: 2 days from the onset of AMI) and just before discharge (median: 15 days from the onset of AMI). The lower detection limit of this test was 0.06 mg/dl.

Blood samples were analyzed in the hospital central laboratory in a blinded fashion.

Follow-up Study

A follow-up study by reviewing medical records or by telephone interview was carried out for all patients. The endpoints were death from any cause and major adverse cardiovascular events (MACE), which included cardiac death, nonfatal MI, heart failure, and the need for percutaneous coronary intervention (PCI) or coronary artery bypass surgery (CABG). The follow-up period for each patient was calculated from the onset of AMI.

Statistical Analysis

Means are expressed with SD for continuous variables, and medians are presented with 25–75th percentiles for skewed variables. Differences in categorical variables between 2 groups were evaluated with χ^2 test. Differences between means or medians for continuous variables were evaluated with t-test or Mann-Whitney U-test, as appropriate. Survival and event-free survival curves were analyzed by the Kaplan-Meier method and comparison between curves was carried out by log-rank test. Multivariate analysis of death and MACE was evaluated with Cox's proportional hazard model. Results were considered significant when the p-value was <0.05. Statistical analysis was performed with StatView 5.0 software (SAS Institute, Cary, NC, USA).

Results

Prevalence and Characteristics of Patients With MeS

Among the 461 AMI patients (326 men, 135 women), 172 had MeS (Fig 1A), a prevalence of 37%. Patients with MeS were more likely to be young and female and to have a history of systemic atherosclerotic disease than patients

Table 1 Comparison of the Patients' Clinical and Angiographic Characteristics

	MeS(+)	MeS(−)	p value
Age (years)	65.9±11.8	68.2±11.6	0.04
Female, % (n)	35% (61)	26% (74)	0.02
Dysglycemia	61% (105)	18% (52)	<0.01
Hypertriglyceridemia	49% (84)	10% (28)	<0.01
Low HDL-cholesterol	94% (161)	40% (117)	<0.01
Hypertension	86% (148)	54% (156)	<0.01
Obesity (BMI ≥25 kg/m²)	49% (84)	13% (39)	<0.01
Atherosclerotic disease, % (n)	28% (49)	20% (57)	0.04
Smoking, % (n)	73% (125)	70% (201)	0.53
LDL-cholesterol (mmol/L)	3.28 (2.73–3.90)	3.42 (2.75–3.79)	0.56
CRP (mg/dl)	0.54 (0.18–1.57)	0.26 (0.12–0.82)	<0.01
Killip class ≥2, % (n)	15% (26)	15% (43)	0.99
Multivessel disease, % (n)	52% (85)	46% (131)	0.33
Anterior MI, % (n)	43% (73)	48% (139)	0.25
PCI, % (n)	71% (122)	75% (216)	0.39
Stent use, % (n) to no. of PCI	79% (96)	78% (168)	0.42
CABG, % (n)	11% (19)	9% (25)	0.42
Peak CK (U/L)	1,953 (1,125–3,477)	1,841 (973–3,142)	0.43
LVEF <40%, % (n)	31% (53)	30% (85)	0.75
Medications during follow-up period			
Oral hypoglycemics, % (n)	28% (48)	7% (21)	<0.01
Insulin, % (n)	15% (25)	4% (12)	<0.01
β-blocker, % (n)	58% (100)	44% (127)	<0.01
Statin, % (n)	40% (68)	33% (96)	0.16
Aspirin, % (n)	97% (166)	94% (271)	0.28
ACEI, % (n)	65% (112)	57% (164)	0.08
ARB, % (n)	11% (18)	8% (24)	0.50

Data are mean±SD, percentage, or median value (interquartile range).
MeS, metabolic syndrome; HDL, high-density lipoprotein; BMI, body mass index; LDL, low-density lipoprotein; CRP, C-reactive protein; MI, myocardial infarction; PCI, percutaneous coronary intervention; CABG, coronary artery bypass surgery; CK, creatine kinase; LVEF, left ventricular ejection fraction; ACEI, angiotensin-converting enzyme; ARB, angiotensin-receptor blocker.

without MeS (Table 1). However, Killip class, incidence of multivessel coronary artery disease and the proportion of coronary revascularization by PCI or CABG, peak creatine kinase, left ventricular dysfunction and anterior wall MI were similar between the 2 groups.

The CRP data for the acute phase was available for 449 patients (median, 2 days after onset of MI). Because 20 patients died in hospital (including 11 patients who died within 3 days of admission because of cardiogenic shock) and 7 patients did not undergo data sampling just before discharge, the CRP data for the chronic phase was available in 422 patients. Although the serum CRP level in the acute phase was similar between the 2 groups (median CRP level of patients with MeS: 1.2 mg/dl vs that of patients without MeS: 1.5 mg/dl, p=0.70), those measured just before discharge (median, 15 days after the onset of infarction) were higher in the patients with MeS than in the patients without MeS (median, 0.54 vs 0.26 mg/dl, p<0.01). As shown in Fig 1B, there was a linear increase in the CRP level as the number of conditions of MeS increased; the median CRP levels for patients with 0, 1, 2, 3, 4, and 5 conditions were 0.19, 0.26, 0.29, 0.43, 0.73, and 1.66 mg/dl, respectively. In the multivariate analysis, CRP level was significantly associated with MeS, but not with infarct size or left ventricular dysfunction.

Medications during the follow-up period, such as insulin, oral hypoglycemic drugs and β-blockers were frequently used in the patients with MeS (Table 1). However, both patient groups similarly received other cardiovascular medications, including statins, aspirin, angiotensin-converting enzyme inhibitors and angiotensin-receptor blockers.

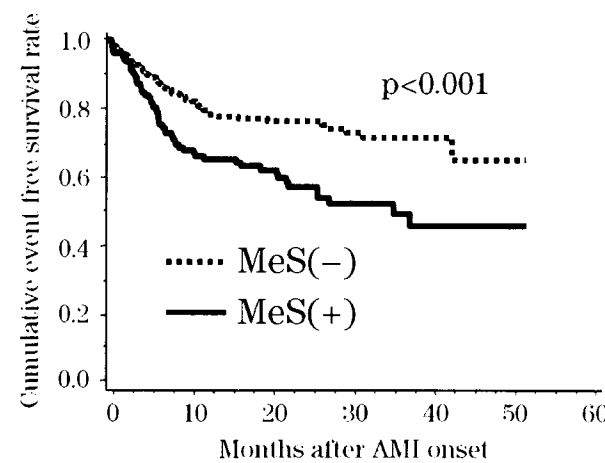

Fig 2. Cumulative event-free survival curves in acute myocardial infarction (AMI) patients with (solid line) and without (dotted line) metabolic syndrome (MeS).

Long-Term Mortality and MACE

During follow-up of a median of 17.6 months (interquartile range: 6.3–30.1), 33 patients died from various causes and 124 patients had at least 1 MACE: cardiac death in 20, heart failure in 24, nonfatal MI in 11 and revascularization in 69 patients. Regarding the occurrence of MI, there were 13 cases of fatal and non-fatal MI during the follow-up. Fig 2 shows that the cumulative-event-free survival rate of patients with MeS was significantly lower than that of the patients without MeS. In the unadjusted Cox's proportional hazard model analysis, the hazard ratio (HR) for MACE in

Table 2 Multivariate Analysis of Predictors of Clinical Outcome

	Death		MACE	
	HR (95%CI)	p value	HR (95%CI)	p value
Age >70 years	3.31 (1.13–9.66)	0.02	1.15 (0.77–1.73)	0.48
Female	3.06 (1.27–7.34)	0.01	1.21 (0.79–1.87)	0.36
Multivessel disease	1.29 (0.45–3.63)	0.62	1.35 (0.90–2.02)	0.14
Killip class ≥2	1.86 (0.76–4.71)	0.16	2.10 (1.29–3.43)	<0.01
LVEF <40%	7.06 (2.50–19.9)	<0.001	1.44 (0.96–2.18)	0.07
CRP ≥0.3 mg/dl	5.57 (1.62–19.2)	<0.01	1.41 (0.94–2.07)	0.09
MeS	1.27 (0.54–3.04)	0.58	1.83 (1.24–2.70)	<0.01

HR, hazard ratio; CI, confidence interval; MACE, major adverse cardiac events. Other abbreviations see in Table 1.

Table 3 Prognostic Value of MeS for MACE

MACE	HR (95%CI)	p value
Cardiac death	0.96 (0.30–3.03)	0.95
Fatal and nonfatal MI	0.84 (0.23–2.70)	0.76
Nonfatal MI	1.02 (0.28–3.69)	0.97
CHF (Killip class ≥2)	2.60 (1.01–6.66)	0.04
Coronary revascularization	2.10 (1.27–3.47)	<0.01

Adjusted for age >70 years, female gender, multivessel disease, Killip class ≥2, LVEF <40, and CRP >0.3 mg/dl.
CHF, congestive heart failure. Other abbreviations see in Tables 1,2.

patients with MeS was 2.05. When we performed multivariate Cox's proportional hazard model analysis for several potential confounders, including age >70 years, female gender, Killip class ≥2, multivessel coronary disease, left ventricular dysfunction (LVEF <40%) and CRP >0.3 mg/dl, MeS remained an independent risk factor for MACE (HR 1.83, 95% confidence interval (CI) 1.24–2.70; p<0.01) after adjustment (Table 2). Multivariate analysis also demonstrated that elevated CRP level even in the stable period, appeared to be a potential determinant of death (HR 5.57, 95% CI 1.61–19.2; p<0.01) and of MACE (HR 1.40, 95% CI 0.94–2.07; p=0.09). To further explore the synergic effect of MeS and CRP, we divided the study patients into 4 groups on the basis of the presence or absence of MeS and on the basis of CRP levels less than or greater than 0.3 mg/dl.[4] When setting patients without MeS and with a low CRP level (<0.3 mg/dl) as the reference, the relative risks of future cardiovascular events following AMI were 1.82 (95% CI 1.12–2.95) in patients without MeS and with a high CRP level (≥0.3 mg/dl), 2.24 (95% CI 1.21–4.16) in patients with MeS and a low CRP level, and 2.56 (95% CI 1.56–4.20) in patients with MeS and a high CRP level. These findings suggest there is a synergistic effect between MeS and CRP for experiencing cardiovascular events in patients following AMI.

We then assessed individual cardiac events among the MACE that were associated with MeS. In the multivariate analysis, MeS was significantly associated with repeated coronary revascularization and congestive heart failure (Table 3). Furthermore, we assessed the individual conditions of MeS for MACE and of the 5 components, only dysglycemia was found to be a significant risk factor for MACE after adjustment (Table 4).

Discussion

The major findings of the present study are that MeS is associated with MACE and that a high CRP level is independently associated with mortality after AMI during a 17.6-month follow-up. Moreover, of the 5 conditions of MeS, dysglycemia is the most important factor in mortality and MACE.

The Observatoire des Infarctus de Cote-d'Or Survey demonstrated that the prevalence of MeS in patients with AMI was 46% and that MeS was associated with in-hospital outcome (eg, development of heart failure several days after the onset of AMI).[13] In that survey population (n=633), only 20–25% of patients underwent reperfusion therapy, so the impact of MeS on long-term prognosis (after discharge) remains unknown, particularly in the recent reperfusion era.

In our study, the AMI patients with MeS had a higher incidence of atherosclerotic disease and a higher CRP level measured during the stable period (median, 15 days after onset) than AMI patients without MeS. In patients with AMI, the CRP level reaches its peak approximately 2–4 days after AMI onset[14,15] in accordance with the extent of myocardial necrosis.[16] Independent of infarct size, the CRP level measured in the stable period (25 days after AMI onset) has been reported to be significantly associated with long-term mortality in patients with AMI.[17] Taken together with the present result by multivariate analysis that CRP level measured in the stable period of AMI was associated only with MeS, but not with infarct size and cardiac func-

Table 4 Incidence and Significance of Each Condition of the MeS for Clinical Outcomes

	Prevalence	Death		MACE	
		HR (95%CI)	p value	HR (95%CI)	p value
Dysglycemia	34.2%	2.39 (0.93–6.11)	0.07	1.61 (1.07–2.41)	0.02
Hypertriglyceridemia	24.3%	0.93 (0.23–3.67)	0.91	1.30 (0.84–2.02)	0.24
Low HDL-cholesterol	60.3%	1.64 (0.55–4.92)	0.37	1.38 (0.89–2.15)	0.15
Hypertension	65.9%	1.27 (0.41–4.00)	0.68	0.83 (0.54–1.24)	0.37
Obesity (BMI ≥25 kg/m^2)	26.9%	0.35 (0.11–1.13)	0.08	1.26 (0.84–1.90)	0.09

Adjusted for age >70 years, female gender, multivessel disease, Killip class ≥2, LVEF <40, and CRP >0.3 mg/dl.
Abbreviations see in Tables 1,2.

tion, it appears that systemic inflammation may be part of the pathophysiology of MeS[18] Therefore, an understanding of the interactions between metabolic and inflammatory pathways may be important with regard to secondary prevention of MACE after AMI.

We found that MeS is significantly associated with repeated coronary revascularization and congestive heart failure, a finding that may be related to the development of new coronary stenosis or restenosis during the follow-up period, leading to myocardial ischemia and left ventricular dysfunction. Previous studies also show that a persistent inflammatory response plays an important role in the post infarction remodeling process[16]

As shown in Table 2, dysglycemia, defined as impaired fasting glucose or previous diabetic medication, is the most important among the 5 conditions of MeS, which may be related to a previous observation that the onset of diabetes follows elevated levels of atherosclerotic risk factors[19] Therefore, it is therapeutically important to simultaneously improve glucose intolerance, abnormal lipid metabolism, blood pressure level and inflammation[20-22]

Controversy exists as to whether the MeS is more than the sum of its independent metabolic components. An important finding in the present study is that MeS is closely associated with elevation of CRP. Moreover, MeS seems to have a synergistic effect on CRP. Because inflammation participates centrally in the process of atherosclerosis[23] MeS may indicate a potential risk for future cardiovascular diseases. Further studies are needed to address this issue.

In conclusion, MeS is associated with a high CRP level and is an important predictor for MACE following AMI. This finding suggests the need for early identification and medical intervention of this underlying disease for preventing future adverse cardiac events.

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Low-dose GH supplementation reduces the TLR2 and TNF-α expressions in visceral fat

Yoshitaka Kubota [a], Hiroyuki Unoki [b], Hideaki Bujo [c,*], Naoaki Rikihisa [a], Akikazu Udagawa [a], Shinya Yoshimoto [a], Masaharu Ichinose [a], Yasushi Saito [d]

[a] *Department of Plastic and Reconstructive Surgery, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[b] *Department of Applied Translational Research, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[c] *Department of Genome Research and Clinical Application, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*
[d] *Department of Clinical Cell Biology, Chiba University Graduate School of Medicine, Chiba 260-8670, Japan*

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Abstract

The increased population of TLR2/TNF-α co-expressing adipocytes is associated with the development of insulin resistance. We have herein shown the significance of low-dose growth hormone (GH) supplementation for the regulation of TLR2 and TNF-α expressions in visceral fat using different kinds of mouse models fed with a high-fat diet. Low-dose GH supplementation reduced the increased population of TLR2/TNF-α co-expressing adipocytes in high-fat fed mice. The neutralization of IGF-1 abolished the effect of GH supplementation on the TLR2 expression using GH-overexpressing mice. IGF-1, but not GH, inhibited the FFA-induced TLR2 and TNF-α expression in 3T3-L1 cells. Finally, low-dose GH supplementation reduced the TLR2 expression without an obvious change in the visceral fat volume in ob/ob mice. These results indicate that low-dose GH supplementation possibly inhibits the high-fat induced change of the adipocytes to TLR2/TNF-α co-expressing cells through the action of IGF-1.

Keywords: Growth hormone; Toll-like receptor 2; Adipocyte; Visceral fat; Insulin-like growth factor-1

A dysfunction of adipocytes leads to an accumulation of metabolic abnormalities, such as dyslipidemia, hypertension, and glucose intolerance [1]. This functional abnormality is characterized by a disturbance in the cytokine expressions of adipocytes, causing the development of insulin resistance, a pathogenesis of the metabolic syndrome [2]. However, the regulation of cytokine secretion from adipocytes accumulated in visceral regions has not yet been fully elucidated.

We have shown that cultured adipocytes implanted in mesenteric, but not in subcutaneous, regions induce tumor necrosis factor (TNF)-α secretion in mice [3]. The TNF-α expression of visceral adipocytes is accompanied with toll-like receptor (TLR) 2 expression, and the population of TLR2/TNF-α co-expressing adipocytes is drastically induced in mice fed a high-fat diet [4]. These observations suggest that the identification of the regulator(s) for the occurrence of TLR2/TNF-α co-expressing adipocytes may provide a target for the amelioration of insulin resistance in the metabolic syndrome.

Insulin resistance accompanied with visceral fat accumulation is not only observed in the metabolic syndrome, but also in several hormonal disturbances. One such hormonal disturbance is growth hormone (GH) deficiency, which is frequently accompanied by reduced insulin sensitivity and accumulated visceral fat. Recent studies have shown the low-dose supplementation of GH to have a beneficial effect on the treatment of insulin resistance accompanied by aging and/or abdominal obesity, as well as GH deficiency itself. [5–7]. These studies have provided a novel

* Corresponding author. Fax: +81 43 226 2095.
E-mail address: hbujo@faculty.chiba-u.jp (H. Bujo).

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therapeutic possibility for GH in the regulation of insulin sensitivity. On the other hand, the beneficial effect of GH raises a complicated issue to be solved, specifically the development of acromegaly-associated glucose intolerance classically observed in association with GH excess. The obvious difference in the opposite effects of GH on the regulation of insulin sensitivity seems to be largely a function of the plasma GH concentration; low dose GH supplementation may be of benefit for GH deficiency and abdominal obesity, whereas excessive GH production results in pathological acromegaly. The aim of this study is to clarify the effect of low-dose GH supplementation on insulin resistance, particularly through the regulation of TLR2/TNF-α co-expressing adipocytes using cultured adipocytes and animal models of visceral fat accumulation.

Materials and methods

Mice and blood samples. Mice were obtained from Charles River Japan. All work was carried out according to the guidelines of the Animal Care Committees of Chiba University. The levels of plasma human growth hormone (Roche), mouse insulin-like growth factor (IGF)-1 (R&D Systems), and mouse insulin (Morinaga) were measured using ELISA kits. Insulin tolerance test was performed by intra-peritoneal injection of human insulin (Sigma–Aldrich) 0.5 or 2.0 U/kg body weight according to the mice models [8].

Cell culture. 3T3-L1 cells were from the American Type Culture Collection. The differentiation of preadipocytes to mature adipocytes was as described [8]. Cells were treated with DMEM supplemented with 10^{-8} M hGH (Novo Nordisk Pharma) or 10^{-8} M human IGF-1 (Jena Bioscience), with 2% free fatty acids (FFA)-free BSA (Sigma–Aldrich) overnight, and then with a fatty acid mixture composed of 500 μM myristic acid and 500 μM palmitic acid, with 10^{-8} M hGH or 10^{-8} M hIGF-1 in the presence of 2% FFA-free BSA at 37 °C for 8 h.

Implantation of 3T3-L1 cells overexpressing hGH into BALB/c nude mice. Human GH cDNA full clone was obtained by polymerase chain reaction (PCR) using human brain-derived cDNA pool using oligonucleotide primers specific for parts of human GH sequence (5′-GACGGC GATCGCCATGGGCTACAGGCTCCCGGAC-3′ and 5′-ATGCGT TTAAACGAAGCCACAGCTGCCCTCCAC-3′). The cDNA fragment subcloned into pcDNA3.1/Hygro(−), were transfected into 3T3-L1 cells using GeneJammer Transfection Reagent (Stratagene). The cells stably expressing hGH and mock transfected cells by the transfection of pcDNA3.1/Hygro(−) without hGH cDNA were cloned as described [9]. The hGH production in the conditioned medium of 3T3-L1 cells overexpressing hGH was 8.8 ± 0.9 ng/10^6 cells/24 h, whereas it was not detectable in conditioned medium of the mock cells. 3T3-L1 cells overexpressing hGH, or the mock cells were suspended at 4×10^6 cells/250 μl in Matrigel (BD Bioscience) and injected subcutaneously in the back of male BALB/c nude mice (6-week old) as described [10]. The mice were fed with high fat diet from a week after the implantation. Insulin tolerance test were performed by using human insulin (Sigma–Aldrich) 0.5 U/kg, i.p. after fasting for more than 16 h.

Isolation of single adipocytes and flow cytometry. Mesenteric fat tissues or 3T3-L1 adipocytes were collected and digested at 37 °C for 60 min with 1 mg/ml type I collagenase (Nitta Gelatin). The digested tissue was centrifuged at 400 rpm for 4 min. The floating adipocyte fraction was prepared for flow cytometry analysis. Isolated adipocytes (1×10^6 cells) were analyzed with FACS Calibur flow cytometer (BD Bioscience) as described [4].

Anti-IGF-1 antibody treatment in mice. Goat polyclonal antibody against mouse IGF-1 (R&D Systems) or normal goat IgG (R&D Systems) was injected i.p. (0.1 μg/g body weight) into male BALB/c nude mice (6-week old) at weekly intervals starting on the day of the implantation of the established 3T3-L1 cells overexpressing hGH or the mock cells. The mice were started to be fed with high fat diet from a week after the implantation. At 4 weeks after the cell implantation, insulin tolerance test was performed by using human insulin 0.5 U/kg, i.p.

RT-PCR. Quantitative RT-PCR amplifications were performed using TaqMan Gene Expression Master Mix (Applied Biosystems) as described [8]. For TLR2 and TNF-α mRNA quantification, Real-time RT-PCR amplification were performed using TLR2 primers (Mm00442346_m1, Applied Biosystems) and TNF-α primers (MA031450, Sigma Genosys). The quantification of given gene, expressed as relative mRNA level compared with a control, was calculated after normalization to 18s rRNA. All PCRs were performed in an ABI PRISM 7000 sequence system (PE Applied Biosystems.) [11].

Fat volume measurement by computed tomography (CT). From 12 weeks of age, male ob/ob mice were administered either hGH (0.5 mg/kg body weight/day) or equivalent volume of saline via mini-osmotic pumps for 4 weeks. There was no significant difference in body weight between the mice administered hGH and the mice administered PBS. The plasma hGH level was 881 ± 643 pg/ml in the mice administered hGH. After fasting for overnight, abdominal CT was performed using GE Healthcare eXplore Locus MicroCT Scanner (GE Healthcare). Visceral and subcutaneous fat volume was calculated using GE Healthcare eXplore Lucus Microview Software (ver 2.2) (GE Healthcare).

Statistical analysis. The results are shown as means ± SD for each index. Statistical significance was determined by means of the Student's *t*-test or Dunnett's multiple range test followed by ANOVA among several groups. Statistical analyses were conducted by using SPSS software (version 13.0J; SPSS Inc.). All P values quoted are two-tailed. A *P*-value of <0.05 was considered statistically significant.

Results

Low-dose GH supplementation reduces the number of TLR2/TNF-α co-expressing adipocytes in visceral fat

We have previously shown that high fat intake induces an increased number of TLR2/TNF-α-coexpressing adipocytes in mesenteric fat in mice [4]. In order to clarify the effect of low-dose GH supplementation on the increase in the population of TLR2/TNF-α co-expressing cells in the adipocytes of mesenteric fat, we performed a flow cytometry analysis of single adipocytes prepared from the mesenteric fat of high-fat fed mice after hGH administration for 2 weeks. There was no significant difference in body weight between the mice administered hGH (GH group) and the mice administered PBS (control group). The plasma hGH concentration in the GH group was 160 ± 86 pg/ml, which is similar to the GH concentrations in previous studies using low dose GH supplementation [12] (Fig. 1A). The plasma IGF-1 concentrations were higher in the GH group in comparison to those in the control group (Fig. 1B). The blood glucose levels 30 min after insulin loading were decreased in the GH group in comparison to the control group (Fig. 1C). The TLR2 mRNA expression levels in mesenteric fat were significantly decreased in the GH group in comparison to those in the control group, suggesting the inhibitory effect of low-dose GH supplementation on TLR2 expression in visceral adipocytes (Fig. 1D). A flow cytometry analysis of single adipocytes prepared from mesenteric fat showed that the high-fat-induced increase in the population of TLR2/TNF-α co-expressing adipocytes was

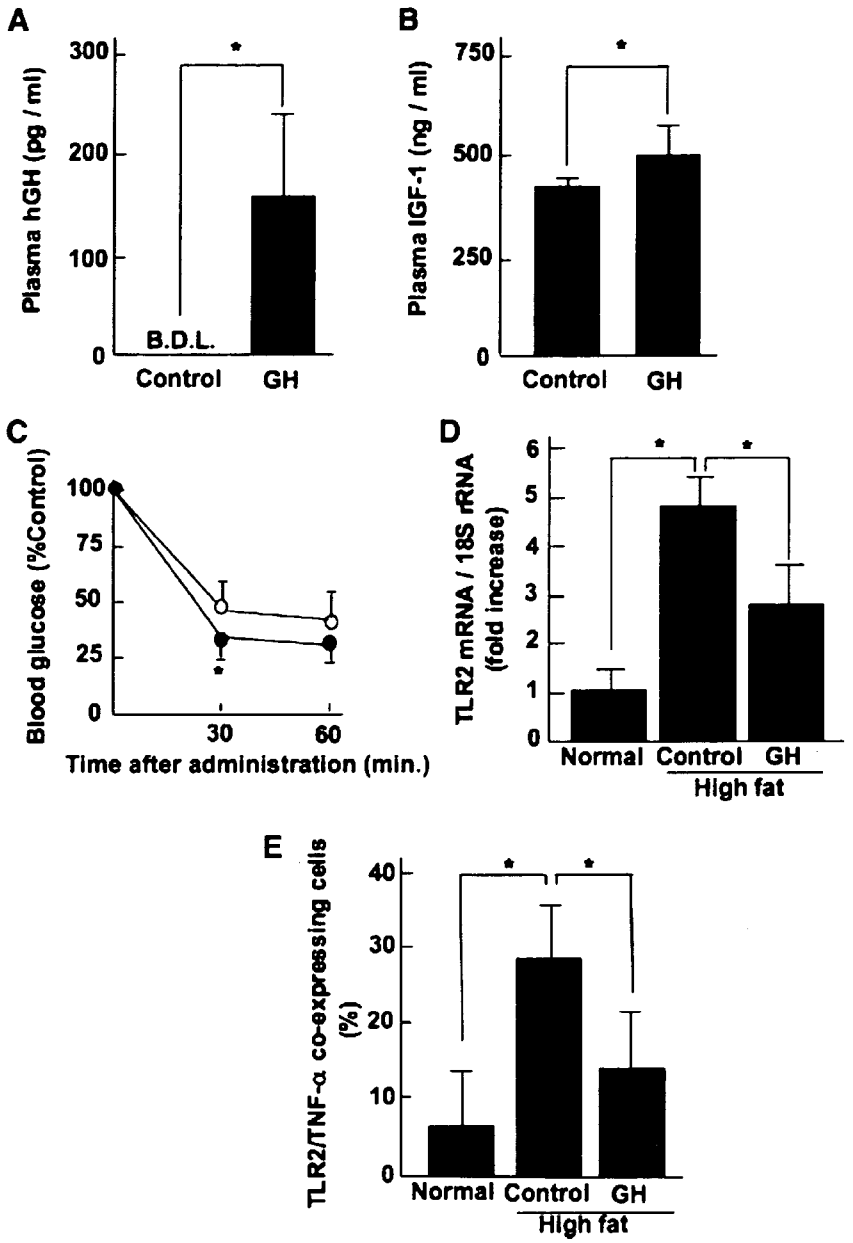

Fig. 1. The effects of low-dose hGH supplementation on reduced insulin sensitivity in high-fat fed mice. (A) The plasma hGH concentration in mice supplemented with hGH (GH) or PBS (control). Male C57BL/6J mice, which have been fed with a high-fat diet (60% fat) for 6 months, were supplemented with hGH (0.05 mg/kg/day) or PBS for 2 weeks. B.D.L., below the detection limit (less than 4 pg/ml). $n = 8$. $^*P < 0.05$ compared to the value of control. (B) The plasma IGF-1 levels in the mice supplemented with hGH (GH) or PBS (control). $n = 8$. $^*P < 0.05$ compared to the value of control. (C) Insulin tolerance test in the mice supplemented with hGH (●) or PBS alone (○). The blood glucose levels were monitored at 0, 30, and 60 min after injection of human insulin. $n = 8$. $^*P < 0.05$ compared to the value of the control. (D) The TLR2 mRNA expression in mesenteric fat tissues of the mice supplemented with hGH (GH) or PBS (control). Normal, mice fed with a normal diet. High fat, mice fed with a high-fat diet. $n = 8$. $^*P < 0.05$ compared to the value of the control. (E) Flow cytometric analyses of TLR2/TNF-α co-expressing adipocytes in the fat tissues of mice supplemented with hGH (GH) or PBS (control). Single adipocytes were prepared from mesenteric fat, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF-α co-expressing adipocytes in the total cells (50,000 cells) were expressed ($n = 8$).

significantly and largely inhibited in the GH group in comparison to that in the control group (Fig. 1E). These results strongly suggest that low-dose GH supplementation reduces the increase in the population of TLR2/TNF-α co-expressing adipocytes in mesenteric fat, as well as reducing insulin resistance, in mice fed a high-fat diet.

Neutralization of IGF-1 abolishes the effect of low-dose GH supplementation on the decrease in the number of TLR2/TNF-α-coexpressing adipocytes

We next analyzed the effect of neutralization of IGF-1, an effector of GH actions for the regulation of insulin sensitiv-

ity, on the decrease in population of TLR2/TNF-α co-expressing adipocytes in visceral fat by low-dose GH supplementation. For this purpose, we established the hGH-expressing mice using cell transplantation methods as described [9]. The hGH-overexpressing 3T3-L1 preadipocytes were subcutaneously implanted into BALB/c nude mice (GH mice). The plasma GH concentrations increased 4 weeks after the implantation of hGH-overexpressing 3T3-L1 preadipocytes were significantly higher in the GH mice than those in the mock-implanted mice (mock mice) (322 ± 165 pg/ml vs 136 ± 128 pg/ml, $P < 0.05$). The plasma IGF-1 concentrations were significantly higher in the GH mice than in the mock mice (323 ± 71 ng/ml vs 267 ± 19 ng/ml, $P < 0.05$) (Fig. 2A). The blood glucose levels 30 min after insulin loading were significantly decreased in the GH mice in comparison to those in the mock mice (Fig. 2B). In accordance with the decreased insulin sensitivity, the plasma triglyceride levels were significantly lower in the GH mice in comparison to those in the mock mice (Fig. 2C). The pretreatment of mice with anti-IGF-1 antibody cancelled the ameliorating effect of GH on the insulin resistance (Fig. 2B). The TLR2 mRNA expression levels of visceral fat were significantly lower in the GH mice than those in the mock mice, and anti-IGF-1 antibody treatment significantly increased the TLR2 mRNA expression levels to those expressed in the mock mice (Fig. 2D). Therefore, circulating IGF-1 is important for the effect of the low-dose GH supplementation on the high-fat-induced insulin resistance in mice.

IGF-1, not GH, inhibits FFA-induced TLR2 and TNF-α gene expressions in 3T3-L1 adipocytes

Two different mouse models fed with a high-fat diet showed that low-dose GH supplementation suppresses the population of TLR2/TNF-α co-expressing adipocytes in visceral fat, and possibly the amelioration by GH supplementation is mediated by the effects of increased plasma IGF-1.

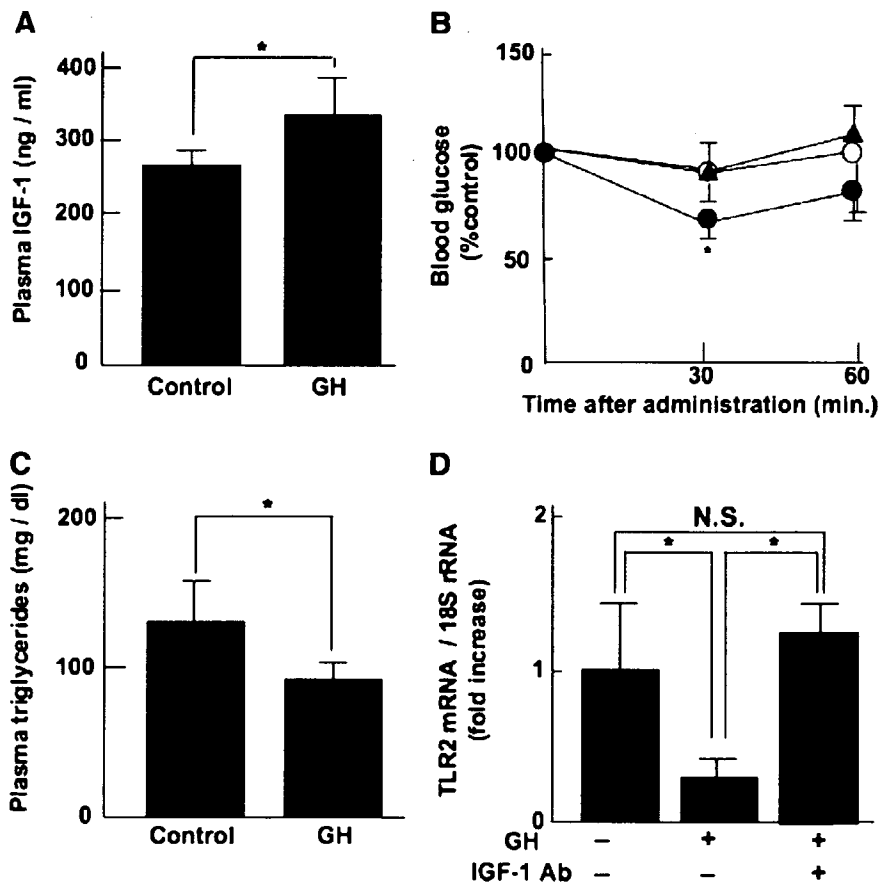

Fig. 2. Effects of IGF-1 neutralization on the actions of low-dose GH supplementation using cell transplantation models. Male BALB/c nude mouse was subcutaneously injected with 10^6 cells of hGH-overexpressing 3T3-L1 preadipocytes. A high-fat diet was started at a week after cell implantation, and continued for 3 weeks. (A) The plasma IGF-1 levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (Control). $n = 6$. $^*P < 0.05$ in comparison to the value of the control. (B) Insulin tolerance test in the mice implanted with hGH-overexpressing cells or with the mock cells (○). The mice implanted with hGH-overexpressing cells were injected with normal goat (●) or anti-mouse IGF-1 antibody (▲). Blood glucose levels were monitored at 0, 30, and 60 min after intraperitoneal insulin injection. $n = 6$. $^*P < 0.05$ compared to the value of the control. (C) The plasma triglyceride levels in the mice implanted with hGH-overexpressing cells (GH) or mock cells (control). $n = 6$. $^*P < 0.05$ compared to the value of the control. (D) TLR2 mRNA expression in mesenteric fat of the mice implanted with hGH-overexpressing cells or PBS alone. Anti-mouse IGF-1 antibody or normal goat IgG was injected after transplantation of hGH-overexpressing cells. $n = 6$. $^*P < 0.05$ compared to the value of the control. N.S., not significant.

Therefore, in order to know the role of IGF-1 in the regulation of TLR2/TNF-α co-expressing adipocytes, we analyzed the effects of IGF-1 on the TLR2 and TNF-α mRNA expressions in 3T3-L1 adipocytes (Fig. 3A). The TNF-α mRNA level was increased by the stimulation of a mixture of myristic and palmitic acids [4]. The incubation of 3T3-L1 cells with hGH did not inhibit the increased expression of TNF-α by FFAs. In contrast, IGF-1 completely inhibited the FFA-induced increase in TNF-α mRNA expression. Furthermore, IGF-1 almost inhibited all of the FFA-induced TLR2 mRNA expression in 3T3-L1 adipocytes. A flow cytometry analysis of single adipocytes prepared from 3T3-L1 adipocytes showed that the FFA-induced increase in the population of TLR2/TNF-α co-expressing adipocytes was largely inhibited by the incubation with IGF-1 (Fig. 3B). These results are in consistent with the observations made using in vivo models (see Figs. 1 and 2), thereby suggesting that IGF-1, not GH, reduces the number of TLR2/TNF-α co-expressing adipocytes in visceral fat.

Low-dose GH supplementation reduces the TLR2 mRNA expression of visceral fat before an obvious change of fat volume in obese mice

We finally examined the effect of low-dose GH supplementation on TLR2 mRNA expression in visceral fat in obese mice in order to know the relationship of TLR2 expression and fat volume in visceral fat. The plasma IGF-1 concentration significantly increased in ob/ob mice supplemented with low-dose GH (GH-ob) in comparison to ob/ob mice in the absence of supplementation (control-ob) (Fig. 4A). Measurements of the fat volume using a CT scan showed no significant difference in either the visceral or the subcutaneous fat volume between the GH-ob and the control-ob mice (Fig. 4B). In contrast, the TLR2 mRNA expression levels of visceral fat tissue were significantly decreased in the GH-ob mice in comparison to those in the control-ob mice (Fig. 4C). These results indicate that low-dose GH supplementation caused the decrease in TNF-α expression in the visceral fat before the obvious change in the visceral fat volume in the obese mice.

Discussion

An abnormal expression of cytokines in adipocytes, particularly in the visceral regions, causes the onset of metabolic syndrome through the development of insulin resistance [2]. We have shown that TNF-α expression is induced in adipocytes accumulated in the visceral, and not in the subcutaneous, regions, using a cell transplantation model [3]. The TNF-α expression in the visceral fat is closely associated with the increased population of TLR2/TNF-α co-expressing adipocytes in response to a high-fat intake [4]. The identification of the TLR2/TNF-

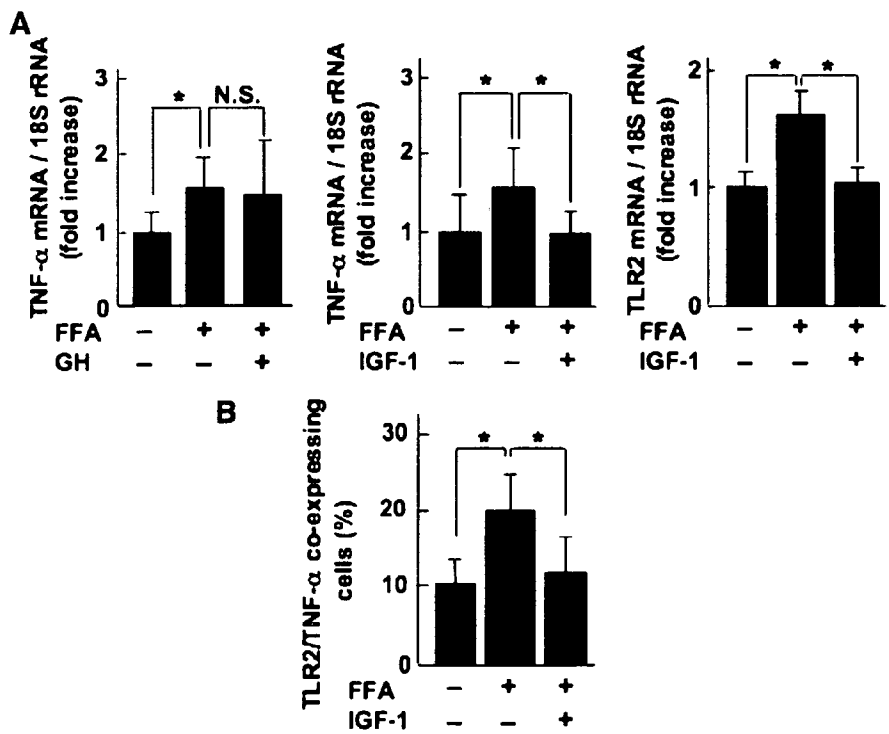

Fig. 3. Effects of GH or IGF-1 on FFA-induced TLR2 and TNF-expressions in 3T3-L1 adipocytes. (A) Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of hGH or IGF-1 for 8 h. Quantitative RT-PCR was used to measure the expression level of TNF-α gene or TLR2 gene $n = 6$. $^*P < 0.05$. (B) Flow cytometric analyses of TLR2/TNF-α co-expressing adipocytes in 3T3-L1 adipocytes. Serum-starved 3T3-L1 adipocytes treated with 1 mM FFA in the presence or absence of IGF-1 for 8 h, and analyzed by FACS Calibur. The averaged populations of TLR2/TNF-α co-expressing adipocytes in the total cells (20,000 cells) were expressed ($n = 3$). $^*P < 0.05$.

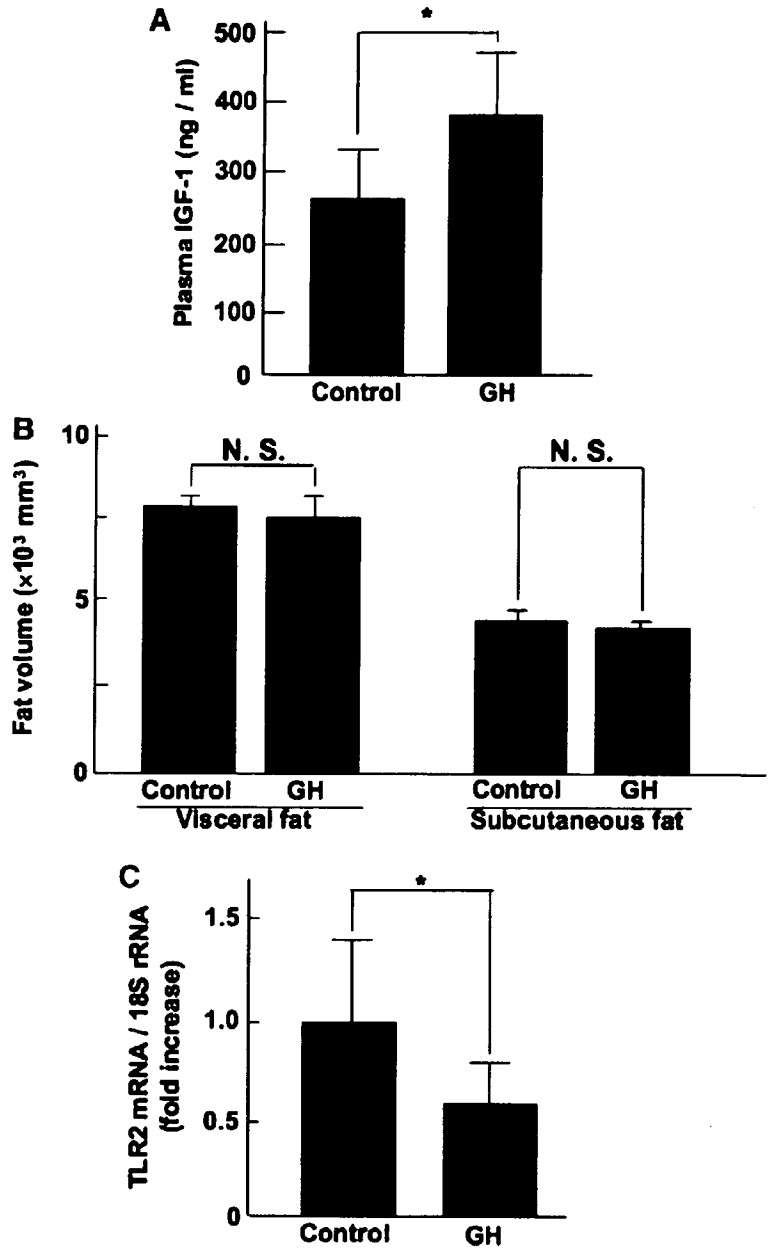

Fig. 4. Effects of low-dose hGH supplementation on the fat volume and TLR2 mRNA expression of visceral fat. Male ob/ob mice were supplemented with hGH (0.5 mg/kg/day) (GH) or PBS (Control) for 4 weeks. (A) The plasma mouse IGF-1 concentration was measured. $n = 7$. $^*P < 0.05$. (B) Visceral or subcutaneous fat volumes at 4 weeks after administration of hGH or PBS alone was measured by using CT. $n = 7$. $^*P < 0.05$. (C) The TLR2 mRNA expression levels in mesenteric fat tissue were measured by RT-PCR. $n = 7$. $^*P < 0.05$.

α co-expressing adipocytes as a regulator of TNF-α expression in the visceral fat suggested that the regulation of the occurrence of pathogenic adipocytes in the visceral fat is important for the improvement of TNF-α-mediated insulin resistance.

Low-dose GH supplementation research has recently focused on the regulation of insulin resistance accompanied by visceral obesity. Yuen et al. found that low-dose GH therapy (0.1 mg/day) improved insulin sensitivity in GH-deficient adults and also notably in subjects with the metabolic syndrome [7]. Johansson showed that GH treatment of obese men reduces the abdominal fat mass, and improved the accompanied metabolic abnormalities [6]. These clinical studies indicate that low-dose GH supplementation is potentially beneficial for metabolic abnormalities accompanied by visceral obesity, in contrast to the glucose intolerance due to the GH overproduction in acromegaly. In this context, there are relevant studies regarding the heterogenous effect of GH on metabolic abnormalities using animal models [13,14]. Based on this background, we performed this study in order to clarify the mechanism for the effect of low-dose GH supplementation on insulin resis-

tance, particularly through the regulation of the population of TLR2/TNF-α co-expressing adipocytes, which has been shown to be related to high-fat-induced insulin resistance [4]. A flow cytometry analysis clearly showed that continuous low-dose GH supplementation reduced the population of TLR2/TNF-α co-expressing adipocytes in visceral regions, and improved insulin resistance. These results using high-fat fed mice are inconsistent with the above clinical observations in obese subjects [6]. We then studied the mechanism of low-dose GH supplementation-mediated inhibition of high-fat induced TLR2 and TNF-α expressions in visceral fat using another model. The GH continuously supplemented from the subcutaneously implanted cells reduced the high-fat induced insulin resistance, and the effect was abolished by the neutralization of IGF-1, a mediator of GH action [15]. The cancellation of GH-mediated action was also observed in the inhibition of TLR2 expression in the visceral fat. Thus, our study showed that IGF-1 was a key molecule in the low-dose GH supplementation for the regulation of TLR2 and TNF-α expressions in visceral fat. The results obtained from cultured adipocytes supported the role of IGF-1 in the effect of low-dose GH supplementation.

The effect of IGF-1 on apoptosis and adipogenesis have been shown in primary cultured adipocytes [16,17]. Our results suggested that TLR2 is one of the genes regulated by IGF-1 in 3T3-L1 cells. The induction of TLR2 expression in high-fat intake could be protected by low-dose GH supplementation through the effect of IGF-1 on visceral adipocytes. The study using ob/ob mice suggested that the effect of IGF-1 on the suppression of TLR2 expression is not necessarily linked to the changes in visceral fat volume. The identification of IGF-1 as a regulator of TLR2 mRNA expression in adipocytes may contribute to the elucidation of the heterogenous functions of GH in various metabolic states. Recent clinical trials suggested that the effects of low-dose GH supplementation are mediated by its ability to increase IGF-1 without the induction of lipolysis [18]. The studies of IGF-1-mediated function on visceral adipocytes may be important for the further therapeutic application of low-dose GH (or IGF-1) supplementation in patients with metabolic syndrome and insulin resistance.

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A Secreted Soluble Form of LR11, Specifically Expressed in Intimal Smooth Muscle Cells, Accelerates Formation of Lipid-Laden Macrophages

Kenji Ohwaki, Hideaki Bujo, Meizi Jiang, Hiroyuki Yamazaki, Wolfgang J. Schneider, Yasushi Saito

Objective—Macrophages play a key role in lipid-rich unstable plaque formation and interact with intimal smooth muscle cells (SMCs) in early and progressive stages of atherosclerosis. LR11 (also called sorLA), a member of low-density lipoprotein receptor family, is highly and specifically expressed in intimal SMCs, and causes urokinase-type plasminogen activator receptor-mediated degradation of extracellular matrices. Here we investigated whether the secreted soluble form of LR11 (solLR11) enhances adhesion, migration, and lipid accumulation in macrophages using animal models and cultured systems.

Methods and Results—Immunohistochemistry showed solLR11 expression in thickened intima of balloon-denuded rat artery. Macrophage infiltration into the cuff-injured artery was markedly reduced in LR11-deficient mice. In vitro functional assays using THP-1–derived macrophages showed that solLR11 (1 μg/mL) significantly increased acetylated low-density lipoprotein uptake by THP-1 cells and cell surface levels of scavenger receptor SR-A 1.7- and 2.8-fold, respectively. SolLR11 dose-dependently increased the migration activity of THP-1 macrophages and adhesion to extracellular matrices 2.0- and 2.1-fold, respectively, at 1 μg/mL. These effects of solLR11 were almost completely inhibited by a neutralizing anti-urokinase–type plasminogen activator receptor antibody.

Conclusion—SolLR11, secreted from intimal SMCs, regulates adhesion, migration, and lipid accumulation in macrophages through activation of urokinase-type plasminogen activator receptor. The formation of lipid-laden macrophages in atherosclerotic plaques possibly is regulated by SolLR11 of intimal SMCs. (***Arterioscler Thromb Vasc Biol.* 2007;27:1050-1056.)**

Key Words: atherosclerosis ■ foam cells ■ macrophages ■ scavenger receptors ■ smooth muscle cells

The early recruitment of monocytes to the arterial neointima, their subsequent differentiation to macrophages, and lipid accumulation are key events in the pathogenesis of atherosclerosis.[1,2] Coincidentally, smooth muscle cells (SMCs) migrate and accumulate in the developing neointimal lesion, where intimal SMCs secrete extracellular matrices, such as elastin, collagen and proteoglycans, inflammatory cytokines, and several proteases.[3,4]

Recent functional studies using genetically modified animals or cells have revealed that certain receptors belonging to the family of low-density lipoprotein (LDL) receptor relatives (LRs) are important regulators of migration, proliferation, and secretory functions of SMCs.[5–10] We have demonstrated that LR11 is abundantly and specifically expressed in intimal SMCs during intimal thickening in a variety of experimental models of atherogenesis, and that its expression is elevated in early stages of neointimal formation.[11–13] LR11 enhances the migration of SMCs by increasing cell-surface urokinase-type plasminogen activator (uPA) receptor (uPAR) levels. LR11 is secreted in soluble form from isolated cultured SMCs, especially in the logarithmic growth phase, and tumor necrosis factor-α converting enzyme is responsible for the shedding of the large ectodomain of LR11.[14,15] This secreted soluble form of LR11 has biological activity toward SMC migration, different from that of the membrane-bound form.[11,16] This finding strongly suggested a solLR11-mediated interaction of intimal SMC and other players, particularly macrophages, in the intima. However, the role of intimal SMCs in the process of lipid accumulation in macrophages has not been well characterized.

The uPAR on monocytes/macrophages is implicated in the pathological infiltration of monocytes into the intima and in the process of foam cell formation.[17,18] Cell-surface expression of uPAR is significantly elevated in monocytes of subjects with acute myocardial infarction and contributes to enhanced cell adhesion in vitro.[17] In apoE$^{-/-}$ mice, overex-

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From the Departments of Clinical Cell Biology (K.O., Y.S.) and Genome Research and Clinical Application (H.B., M.J.), Chiba University Graduate School of Medicine, Chiba, Japan; Kowa Research Institute (H.Y.), Kowa Co Ltd, Higashimurayama, Japan; Department of Medical Biochemistry (W.J.S.), Max F. Perutz Laboratories, Medical University of Vienna, Vienna, Austria.

Correspondence to Hideaki Bujo, Departments of Genome Research and Clinical Application Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan. E-mail hbujo@faculty.chiba-u.jp

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pression of human uPAR in macrophages enhances cell adhesion to the aortic wall,[18] and targeted overexpression of uPA, a ligand of uPAR, in macrophages accelerates atherosclerosis with increased foam cell formation.[19]

Thus, solLR11 might be expected to modify the macrophage foam cell formation through the activation of uPAR-mediated extracellular matrix degradation. Here we demonstrate the presence of solLR11 in hyperplastic intima, and show that solLR11 deficiency drastically reduces the infiltration of lipid-laden macrophages into the intima of LR11$^{-/-}$ mice on a high-fat diet using a cuff-injury model. Cell culture experiments showed that recombinant solLR11 increases the migration and adhesion of macrophages to extracellular matrix and SMCs through enhanced expression of adhesion molecules, as well as lipid accumulation through scavenger receptors. These results support a novel function of intimal SMCs in the regulation of macrophage-foam cell formation in the process of atherosclerosis.

Materials and Methods

Antibodies and Cells

Preparation and properties of the monoclonal and polyclonal antibodies against human and mouse LR11, 5-4-30-19-2 and pm11, respectively, were described previously.[11] Monoclonal antibodies against SR-A (KT022) was obtained from Wako (Tokyo, Japan). Polyclonal or monoclonal antibodies against uPAR (AF807), VLA-4 (BBA37) and P-selectin glycoprotein ligand (PSGL)-1 (MAB996) and recombinant platelet-derived growth factor (PDGF)-BB (520-BB) were from R&D systems (Minneapolis, Minn). Monoclonal antibody against Mac-3 was from BD Pharmingen (San Diego, Calif). Primary cultures of SMCs were prepared from the isolated medial layer of rat aortas as described.[20] COS7 cells were from ATCC (CRL-1651; Manassas, Va). THP-1 cells were obtained from ATCC (TIB-202) and maintained in RPMI 1640 containing 10% fetal bovine serum. THP-1 cells were differentiated to macrophages (THP-1 macrophages) by treatment with 200 nM of phorbol 12-myristate, 13-acetate (PMA; Promega, Madison, Wis) for 24 hours at 37°C in the presence or absence of purified solLR11 at 1 μg/mL (unless indicated otherwise) and/or of the indicated antibodies.

Animal Experiments

All animal studies were reviewed and approved by the animal care and use committee of the Stockholm Animal Ethics Board. Male Wistar rats (Charles River Laboratories, Chiba, Japan), weighing 400 to 450 grams, were anesthetized, and the left common carotid artery was denuded by ballooning as described.[21] The left carotid arteries were isolated at 7 or 14 days after injury and used for histochemical staining, immunohistochemistry and Western blot. Female LR11$^{-/-}$ and LR11$^{+/+}$ mice, aged ≈40 weeks fed a high-fat diet (Research Diets, Inc; 60 kcal% fat supplied from lard and soybean oil, 20 kcal% carbohydrate from sucrose and maltodextrin, and 20 kcal% protein from casein) from 3 days before surgery, were anesthetized, and the left femoral artery was sheathed with a polyethylene cuff made of PE90 tubing as described.[11] then maintained on high-fat diet. The left femoral arteries were isolated at 7 days after cuff placement and used for histochemical staining and immunohistochemistry.

Generation of Knockout Mouse

LR11$^{-/-}$ mice were generated as described (Jiang et al, submitted). Briefly, an LR11 targeting vector was constructed with short (3.3 kb) and long (4.4 kb) arms of homology and a Neo cassette (3.9 kb) to target the first exon of mouse LR11. Cultured embryonic stem cells were transfected with the LR11 targeting vector, homologous recombinant clones were selected with G418, and confirmed by Southern blotting. Germline-transmitted chimeras obtained were crossbred with C57BL6/J females, and resulting heterozygous offspring were interbred. Wild-type, heterozygous, and homozygous mutant mice were born in Mendelian ratios. All mice born were maintained under standard animal house conditions with a 12-hour light/dark cycle and were fed ad libitum with regular chow diet.

Immunohistochemistry and Western Blot

Serial paraffin-embedded sections (5 μm) were used for immunohistostaining as described.[12] Briefly, sections were pretreated with 3% H_2O_2 to inactivate endogenous peroxidase. Slides were then stained with anti-LR11 (pm11, 1:50) or anti-Mac3 (1:25) for 1 hour at 25°C in the presence of 0.1% bovine serum albumin. Vectastain ABC-AP kit (Vector Laboratories) was used with biotin-conjugated anti-mouse IgG or anti-rabbit IgG secondary antibodies (Wako) according to the manufacturer's instructions. Slides were counterstained with hematoxylin-eosin and elastica van Gieson. Western blot analysis was performed as described previously[22] using anti-LR11 (pm11, 1:500), anti-VLA-4 (1:250), anti-SR-A (1:250) and anti-uPAR (1:250).

Construction, Expression, and Purification of SolLR11

Materials and Methods for this study are fully described in the online data supplement section (please see http://atvb.ahajournals.org). Briefly, we first constructed an expression plasmid for the soluble form of LR11 lacking 104 C-terminal amino acids containing the transmembrane region. COS7 cells were transfected with the expression construct and solLR11 was purified using Ni^{2+}-chelating chromatography. The biological activity of purified solLR11 was confirmed by a SMC migration assay.[13]

Adhesion and Migration

Cell adhesion was determined in 96-well plates as described.[23] Wells were coated with 5 μg/mL collagen or fibronectin for 2 hours at 37°C. THP-1 macrophages were fluorescently labeled by loading with Calcein-AM dye for 1 hour at 5×10^6 cells/mL in RPMI containing 1% fetal bovine serum. Calcein-loaded cells were then added to the extracellular matrix coated plates at 2.5×10^5 cells/well, and incubated for 30 minutes at 37°C. Nonadherent cells were removed by gently washing with phosphate-buffered saline, and adherent cells were analyzed by measuring fluorescence using a fluorescence microplate reader, SPECTRAmax GEMINI XS (Molecular Devices, Menlo Park, Calif). Cell migration was measured in a 96-well micro-Boyden chamber with collagen type I-coated filters as described.[13] The lower chamber contained RPMI 1640 with 5 ng/mL PDGF-BB, and THP-1 macrophages were added to the upper chamber and incubated for 4 hours at 37°C. Migrated cells were quantitated using a fluorescence microplate reader.

Acetyl-LDL Uptake

THP-1 macrophages were seeded on 96-well culture plates and incubated with the indicated concentrations of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)-labeled acetylated LDL (DiI-AcLDL) for 4 hours at 37°C. Then, unincorporated DiI-AcLDL was removed by washing with phosphate-buffered saline. DiI-AcLDL uptake was measured using a fluorescence microplate reader.

Statistics

The results are shown as mean±SD for each index. Comparison of data were performed using the Student t test or Williams test; $P<0.05$ was considered significant.

Results

LR11, Expressed in Intimal SMCs, Is Secreted as a Soluble Form in the Intima of Balloon-Denuded Artery

A soluble form of LR11 is secreted from cultured SMCs and induces the migration activity of SMCs together with the

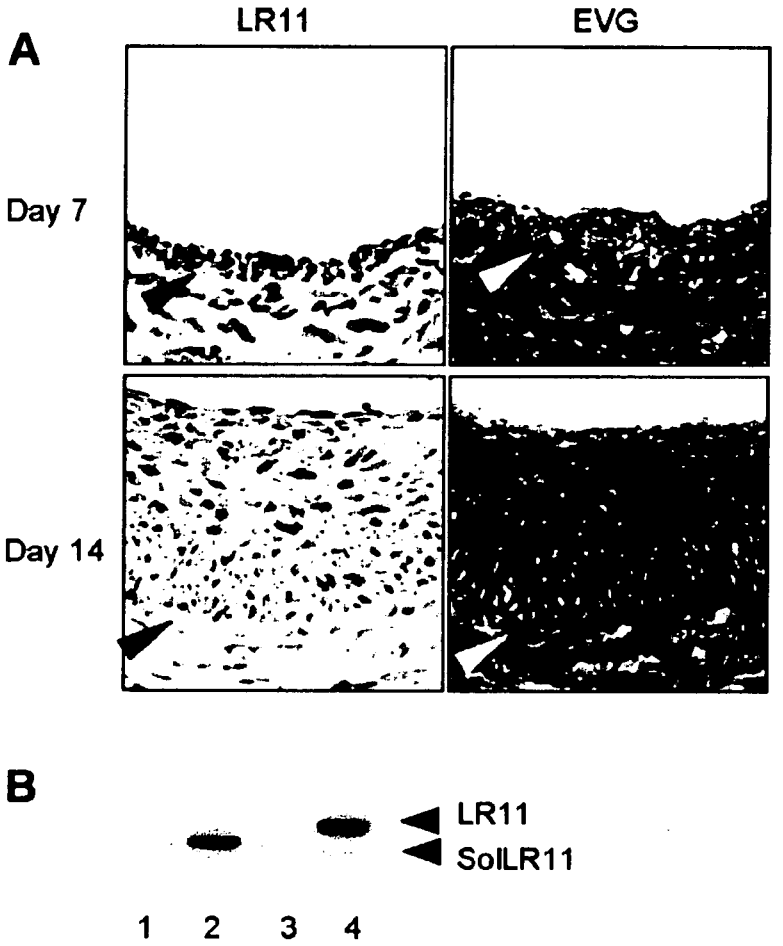

Figure 1. SolLR11 expression in intimal SMCs in balloon-denuded rat artery. A, Sections of balloon-denuded carotid artery were subjected to histological analysis using elastica van Gieson staining (EVG), and to immunohistochemistry with anti-LR11 antibody (pm11) at day 7 (top) and day 14 (bottom) after injury. Arrowheads indicate the internal elastic layers. B, Intima from day 14 balloon-denuded carotid artery was homogenized and analyzed by Western blotting with anti-LR11 antibody (pm11). Ln 1: mock/COS7; lane 2: solLR11/COS7; lane 3: medial layer extract; lane 4: intimal layer extract. Arrowheads indicate the full-length and truncated soluble LR11, respectively.

membrane-anchored form.[11] To investigate the pathophysiological relevance of solLR11 in the process of neointimal formation, the expression of soluble and membrane-anchored LR11 proteins were analyzed in the rat balloon injury model. Immunohistochemistry and Western blot showed that LR11 is highly and specifically expressed in intimal SMCs, and that its expression is higher at day 7 after injury than at day 14 (Figure 1A). This is in agreement with the finding that LR11 is specifically expressed in the proliferating phase of SMCs in culture.[11] Using the samples of thickend intima obtained at day 14, secreted solLR11 with reduced molecular size compared with that of membrane-bound LR11, was detected in intimal homogenates, as expected from the results in cultured SMCs (Figure 1B).

Macrophage Infiltration and Lipid Accumulation in Intima of Cuff-Injured Artery Is Inhibited in LR11 Knockout Mice

Blocking LR11's function by neutralizing antibody significantly reduced neointimal thickening in cuff-injured femoral artery in mice.[11] We have recently established LR11 knockout mice, in which the coronary arterial structure appears histopathologically normal (Jiang et al, submitted). To clarify the role of solLR11 in neointimal formation, we applied cuff injury in femoral artery in the LR11 $^{-/-}$ mice on a high-fat diet. Infiltration of Mac3-positive macrophages and lipid accumulation in macrophages were detected at 7 days after cuff placement, and elastin-rich neointimal thickening was observed at day 28 in wild-type mice on a hig- fat diet (Figure 2). The intimal thickness at day 28 after cuff injury was significantly reduced in the LR11 $^{-/-}$ mice compared with the mice on normal chow diet (Jiang et al, submitted). Surprisingly, infiltration of Mac3-positive and lipid-laden macrophages was significantly decreased in the SMC-rich early neointima. These data suggest that LR11 is involved in lipid accumulation and macrophage infiltration into the intima at an early stage of injury-induced neointimal formation.

Expression, Purification, and Biological Activity of Recombinant SolLR11

To investigate the mechanism of decrease in intimal lipid-laden macrophages after cuff injury, we analyzed the effect of solLR11 on macrophages using the established cell line, THP-1. Recombinant solLR11 was expressed using a COS7 expression system and purified by single step Ni^{2+}-chelating chromatography (supplemental Figure I, available online at http://atvb.ahajournals.org). The addition of purified recombinant solLR11 at 1, 10, and 100 μg/mL strongly increased the PDGF-induced migration activity of SMCs when compared with SMCs transfected with vector alone or vector containing full-length LR11 (supplemental Figure I). The enhancement of SMC's migrating activities by LR11s were completely blocked by anti-LR11 antibody.

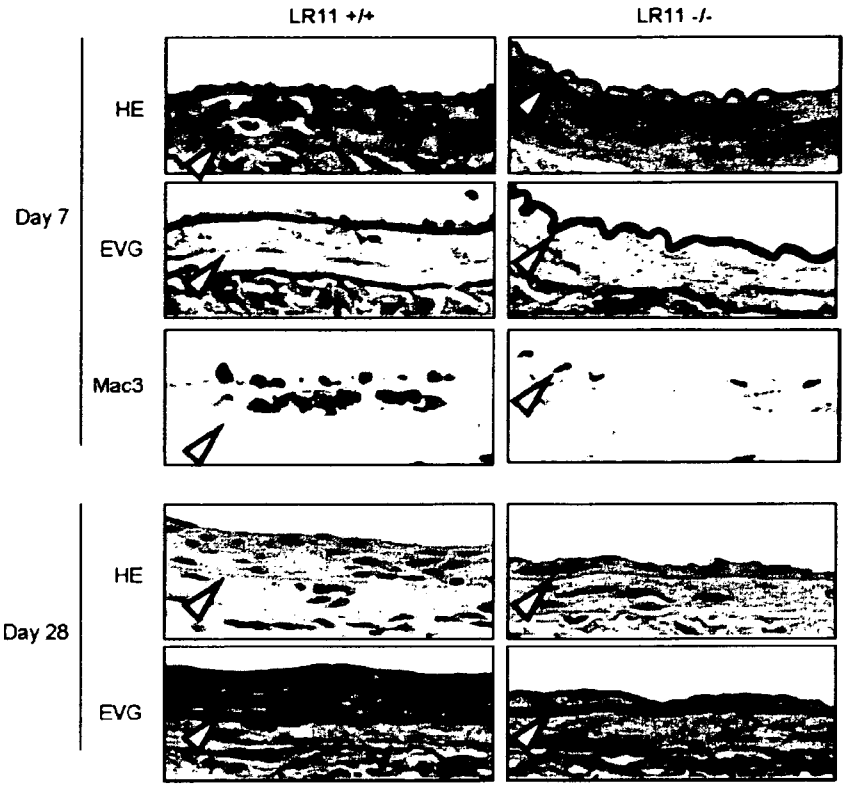

Figure 2. Reduced macrophage infiltration into intima of cuff-injured artery in LR11$^{-/-}$ mice. Sections of femoral artery obtained at day 7 and day 28 after cuff injury in LR11$^{-/-}$ and LR11$^{+/+}$ mice on a high-fat diet were subjected to histological analysis using hematoxylin & eosin (HE) and EVG staining, and to immunohistostaining with anti-Mac3 antibody. Arrowheads indicate the internal elastic layers.

SolLR11 Increases Scavenger Receptor Expression and Lipid Accumulation in THP-1 Macrophages

Because LR11KO mice showed reduced lipid-containing macrophages (Figure 2), we next investigated the effect of solLR11 on the regulation of scavenger receptor expression and lipid accumulation of THP-1 macrophages. THP-1 macrophages were cultured for 24 hours in the presence or absence of PMA and/or solLR11 at 1 μg/mL, followed by Western blot of plasma membrane preparations probed with anti-SR-A and anti-uPAR antibodies. Although solLR11 did not induce SR-A protein expression in the absence of PMA, it increased SR-A expression 2.8-fold in its presence (Figure 3A). The cell-surface level of uPAR was increased by solLR11, likely because of the solLR11-mediated stabilization of uPAR.[11] To test whether solLR11 affects lipid accumulation in macrophages, we evaluated DiI-AcLDL uptake in THP-1 macrophages (Figure 3B). In the undifferentiated THP-1 cells, there was no significant DiI-AcLDL uptake, and solLR11 did not affect DiI-AcLDL uptake (data not shown). However, in THP-1 macrophages, solLR11 at 1 to 100 μg/mL significantly increased DiI-AcLDL uptake (Figure 3C). Addition of neutralizing anti-LR11 or anti-uPAR antibodies almost totally inhibited the increase in DiI-AcLDL uptake by the cells (Figure 3D). These data indicate that solLR11 stimulates lipid uptake via SR-A, and that the accelerated lipid accumulation in macrophages may be attributable to the LR11-mediated upregulation of uPAR levels.

Recombinant SolLR11 Increases Adhesion and Migration of THP-1–Derived Macrophages

We next investigated the effect of solLR11 on the adhesion of THP-1–derived macrophages (THP-1 macrophages) in vitro using the recombinant protein. THP-1 cells were differentiated to macrophages by the treatment with 200 nM PMA for 24 hours, and then the cells were labeled with fluorescent dye Calcein-AM for quantitative analysis by the in vitro adhesion assay. SolLR11 at 1 μg/mL significantly increased the adhesion of THP-1 macrophages to collagen and fibronectin (Figure 4A) 1.8- and 2.1-fold, respectively. The neutralizing anti-LR11 antibody completely blocked solLR11-induced increase in adhesion. Next, we tested the effect of solLR11 on the adhesion of macrophages to SMCs, because of the drastic decrease in macrophage recruitment in intima of cuff-injured artery in LR11$^{-/-}$ mice, principally caused by proliferating SMCs. Pretreatment of THP-1 macrophages with 1 μg/mL solLR11 increased cell adhesion to cultured SMCs 1.6-fold (Figure 4B). The addition of neutralizing antibodies against VLA-4 and PSGL-1 completely inhibited the increased adhesion by solLR11, as observed with anti-LR11 or anti-uPAR antibodies. Thus, we analyzed the effect of solLR11 on the expression of adhesion molecules. SolLR11 enhanced the expression of cell-surface VLA-4 in the presence and absence of PMA (Figure 4C).

We next tested the effect of solLR11 on the migratory functions of THP-1 macrophages by using the Boyden chamber method. SolLR11 itself did not affect migration of THP-1 macrophages in vitro (data not shown). When cells were preincubated with 1 μg/mL solLR11 for 12 hours, PDGF-BB-induced migration of THP-1 macrophage was 2.0-fold greater than in the absence of solLR11 (Figure 4D). The stimulatory effect of solLR11 was decreased by addition of neutralizing anti-LR11 or anti-uPAR antibodies. These data indicate that solLR11 induces adhesion and migration

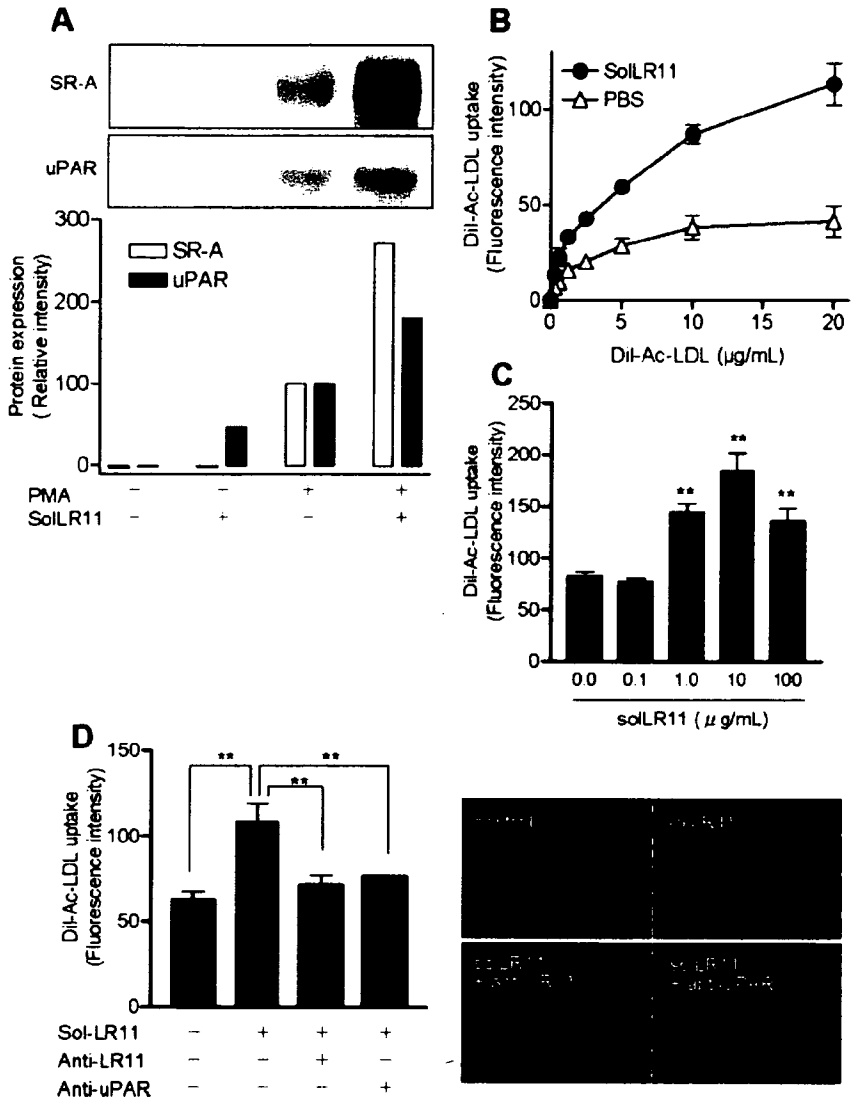

Figure 3. SolLR11 enhances cell-surface expression of SR-A and uPAR, and the uptake of modified LDL by THP-1 macrophages. A, Membranes of THP-1 monocytes or macrophages, prepared from cells obtained under the conditions indicated in the bottom panel, were subjected to Western blotting with anti-SR-A or anti-uPAR antibodies. B, THP-1 macrophages were preincubated with 1 μg/mL solLR11 (black circles) or phosphate-buffered saline (white triangles). Cells were washed and then incubated with the indicated concentrations of DiI-AcLDL in the presence or absence of excess amount of Ac-LDL. C, Dose-dependent effect of solLR11 on DiI-AcLDL uptake by THP-1 macrophages. D, The effects of anti-uPAR and anti-LR11 (5-4-30-19-2, 1:2 dilution) antibodies on solLR11-induced uptake of DiI-AcLDL (10 μg/mL) by THP-1 macrophages. DiAcLDL uptake was visualized by fluorescence microscopy and quantitative measurement was obtained using a fluorescence microplate reader. Data are expressed as mean±SD, n=6 (*$P<0.05$, **$P<0.01$).

activities of macrophages through uPAR-mediated pathways, possibly through increasing the levels of cell-surface adhesion molecules.

Discussion

In this study, we have shown that LR11 is secreted in a soluble form from intimal SMCs in a balloon injury model, and that LR11-deficient mice show drastically decreased lipid-accumulating macrophages in early intimal formation after cuff injury in mice on a high-fat diet. Functional analysis of recombinant solLR11 demonstrated that solLR11 can regulate the functions of THP-1 macrophages toward foam cell formation, such as lipid incorporation, adhesion, and migration. The inducing effect on foam cell formation of solLR11 was almost abolished by functional neutralization of solLR11 or of its target protein, uPAR. Based on these results, we propose a new role of intimal SMCs in the regulation of monocyte/macrophage functions involving the secretion of soluble LR11.

Although LR11 was originally identified as a type I transmembrane protein, significant amounts of LR11 are shed from cultured SMCs, IMR32 and BON cells, and hydra as a soluble form of the large extracellular domain cleaved off by metalloprotease.[11,15,24] In CHO cells, it was demonstrated that tumor necrosis factor-α convertase is responsible for the proteolytic cleavage of LR11.[14,25] However, the physiological function of solLR11 is still poorly understood because of the lack of availability of recombinant protein. We have reported that solLR11, secreted from cultured cells as well as the membrane-bound form,[11] enhance SMC migration, and that the expression of solLR11 largely depends on the differentiation stage of SMCs. The medial contractile type does not express solLR11, whereas the intimal synthetic type does, consistent with the expression of embryonic myosin isoform SMemb.[16] These data suggest that LR11-expressing cells likely perform diverse functions via secretion of soluble LR11 and/or expression of membrane-bound LR11, respectively.

We detected solLR11 protein by Western blot of thickened intima obtained 14 days after balloon injury (when neointimal formation is almost accomplished). Although the level of solLR11 expression was lower than that of the membrane-

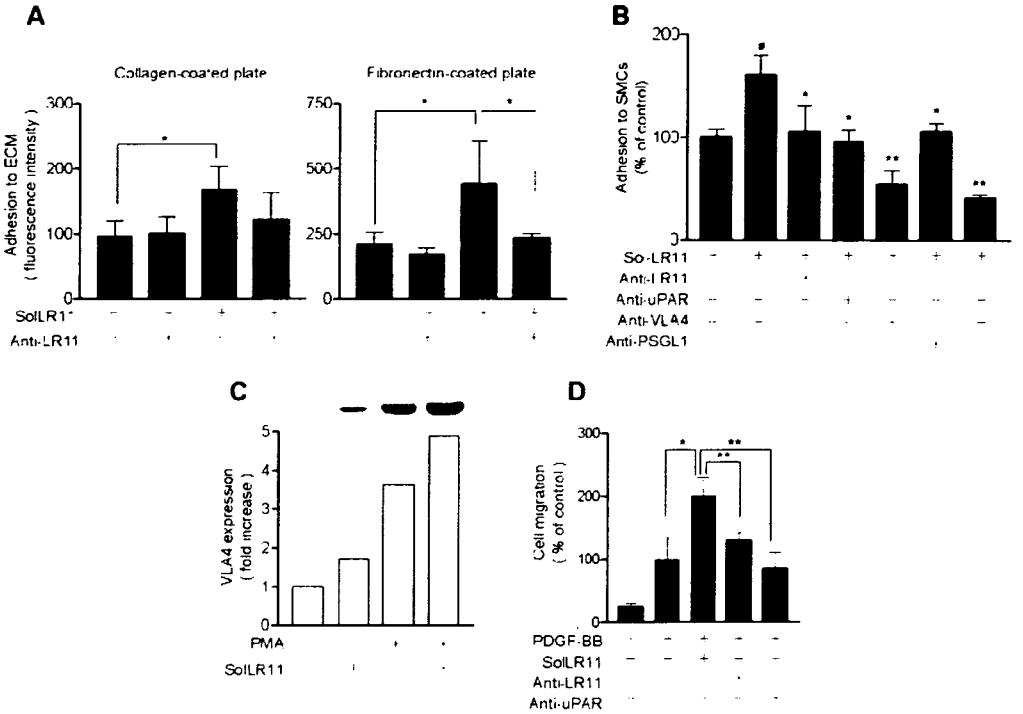

Figure 4. SolLR11 enhances adhesion and migration of THP-1 macrophages. A, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR antibodies. The cells were washed and then incubated on collagen- or fibronectin-coated plates. B, THP-1 macrophages were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-uPAR, anti-LR11 (5-4-30-19-2, 1:2 dilution), anti-VLA-4 or anti-PSGL-1 antibody. The cells were washed and then incubated in the cultured SMCs. C, THP-1 monocytes were treated with 200 nM PMA in the presence or absence of 1 μg/mL solLR11. Membranes were subjected to Western blotting with anti-VLA-4 antibody. D, Cells were preincubated with 1 μg/mL solLR11 in the presence or absence of neutralizing anti-LR11 (5-4-30-19-2, 1:2 dilution) and anti-uPAR antibodies. The cells were washed, and then the PDGF-induced cell migration was measured using a micro-Boyden chamber. Data are expressed as mean±SD, n=4 to 6 (*P<0.05, **P<0.01).

bound form, solLR11's expression at an earlier stage is likely higher than that at late stages, because solLR11 was specifically expressed in rapidly proliferating SMCs in culture.[11] The macrophage infiltration into the intima and lipid accumulation was greatly decreased in LR11 knockout mice compared with those in wild-type mice (Figure 2). Because the expression of LR11 was barely detectable in monocytes/macrophages, we hypothesize that the soluble form of LR11 from intimal SMCs affects macrophage functions that facilitate progression of atherosclerosis, especially in early neointimal formation. With the preparation of recombinant solLR11, we were able to obtain experimental support for our above hypothesis concerning the role of solLR11 in macrophage function.

Macrophages express a variety of scavenger receptors which are involved in uptake of modified LDL and atherogenesis.[2,26,27] SR-A is highly expressed almost exclusively in differentiated macrophages, and is implicated in increased foam cell formation in atherogenesis.[28,29] We showed that solLR11 enhanced SR-A expression and DiI-AcLDL accumulation in THP-1 macrophages in vitro, suggesting a possible role of solLR11 in the formation of lipid-rich plaques. Furthermore, solLR11 significantly enhanced monocyte adhesion not only to extracellular matrices but also to the cultured SMCs in vitro. Increased adhesion and infiltration of circulating monocytes is believed to be the key event in early stage of atherogenesis. Furthermore, the direct association between monocytes and SMCs is implicated in the prolonged retention of monocytes in atherosclerosis, and increases matrix metalloproteinase-1 production, possibly leading to the formation of unstable plaque.[30] Monocyte adhesion to SMCs is mediated, eg, by vascular cell adhesion molecule-1, and immunohistochemical analysis showed the abundant expression of vascular cell adhesion molecule-1 in SMCs in human atherosclerotic lesions.[31] PDGF-BB and angiotensin II are implicated in the enhanced binding of monocytes to cultured SMCs. Thus, solLR11 is probably involved in monocyte accumulation at activated areas in plaques at which SMCs actively migrate and proliferate, and prolongs on-site retention of macrophages.

SolLR11 increased cell-surface uPAR levels in THP-1 monocytes/macrophages. Moreover, solLR11-enhanced lipid uptake, adhesion, and migration of THP-1 macrophages were almost completely blocked by neutralizing anti-uPAR as well as anti-LR11 antibodies. The increased expression of uPAR on monocytes/macrophages is implicated in the adhesion, differentiation, and increased metalloproteinase expression in the cells. Moreover, uPAR expression is increased in circulating monocytes in patients with acute myocardial infarction compared with that in patients with chronic stable angina.[17] LR11 upregulates cell surface uPAR levels in SMCs by inhibition of its catabolism, which is mediated by LRP1,

another member of the LDLR family.[11] LRP1 is also abundantly expressed in monocytes/macrophages;[8] hence, it is likely that LR11 regulates macrophage differentiation and lipid accumulation in plaques by increasing uPAR levels in monocytes/macrophages.

In summary, SMCs and macrophages coexist in plaques throughout the progressive stage of atherogenesis. SolLR11, which is secreted from activated SMCs in the intima, likely is a coregulator of scavenger receptor expression, lipid accumulation, adhesion, and migration of monocytes/macrophages at an early stage of neointimal formation. The uPAR-mediated effects were observed at the same concentration range (0.1 to 10 μg/mL) of recombinant solLR11 in cultured macrophages as that required for the migration of SMCs (Figure 1D). Although the pathophysiological concentrations of solLR11 in intima is difficult to determine, the increase in levels of intimal solLR11 in injured arteries, and the loss of infiltrated macrophages in LR11-KO mice strongly suggest that intimal SMCs locally secrete sufficient amounts of solLR11. Nevertheless, the elucidation of the significance of interactions of SMCs and macrophages involving solLR11 requires further analyses using various models for atherosclerosis. Clearly, the regulation of solLR11 function in the arterial wall is a promising target not only for such studies but also for therapeutic amelioration of atherosclerosis with unstable plaque.

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Disclosures

None.

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