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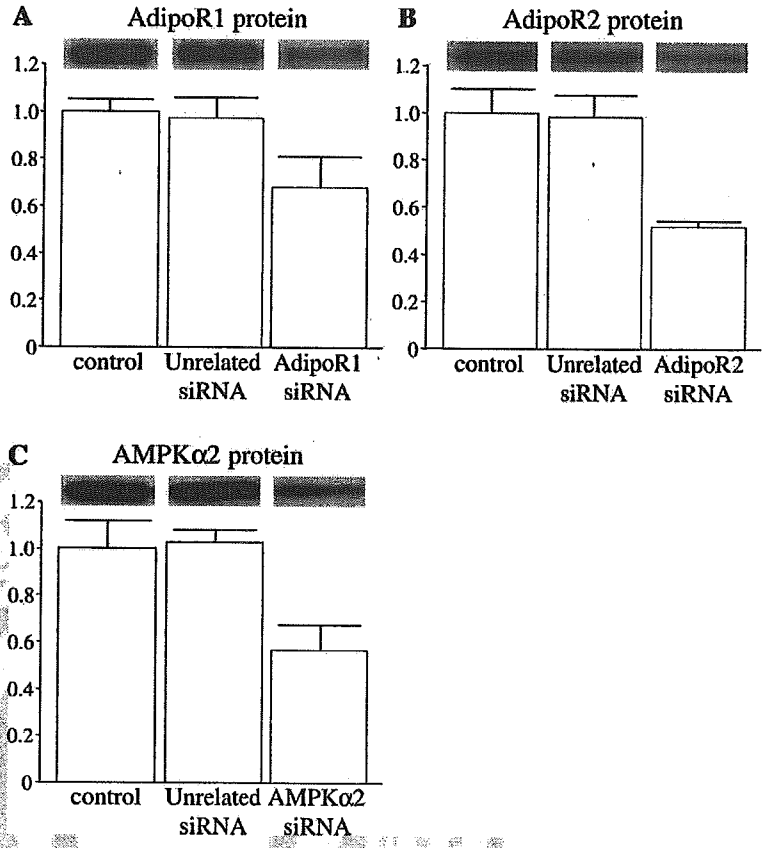

Fig. 4. Effects of siRNA transfection on protein expression levels of AdipoR1 (*A*), AdipoR2 (*B*), and AMPKα_2 (*C*). Cultured cardiomyocytes were transfected with each siRNA, cultures were washed, and extracts of cells were used for immunoblot analysis. Intensity of β-tubulin band was used as a loading control between samples. Protein levels are expressed relative to nontreated control cells (= 1). Values are means ± SE (n = 6). *P < 0.05 vs. control.

NIH Image J analysis software was used to measure surface area of fixed cardiomyocytes. One hundred cells from randomly selected fields in three wells were examined for each condition. The cell surface area was determined in cells pretreated for 4 h with or without adiponectin or AICAR and then incubated with ET-1 (100 nmol/l), ANG II (100 nmol/l), or IGF-I (100 nmol/l) for 48 h.

RNA interference and transfection. Small interfering RNAs (siRNAs) were designed and synthesized by Invitrogen. The sequences of the sense siRNAs are listed in Table 1. The cultured cardiomyocytes were transfected with 270 nM siRNA with use of Lipofectamine 2000. After the cultures were washed, the medium was replaced with DMEM containing 0.5% FCS for 12 h. AdipoR1, AdipoR2, or AMPKα_2 was suppressed with the appropriate siRNA for determination of the effects of adiponectin on AMPK and ACC phosphorylation and ET-1-induced cellular hypertrophy and ERK phosphorylation.

Animal models of myocardial infarction. Myocardial infarction was created in 12- to 16-wk-old male mice and rats by ligation of the left coronary artery under anesthesia with pentobarbital sodium (50 mg/kg ip) and ventilation with a respirator. The chest was closed with 7.0 polypropylene sutures, and the animals were killed and tissues were harvested 2 wk after the surgery.

Parts of the tissue samples from the left ventricle were quickly frozen and stored at −80°C until measurement of mRNA and protein expression levels. Other parts of the samples were fixed in 10% formalin solution and embedded in paraffin and then sliced into 5-μm-thick sections, which were stained by the immunoperoxidase method (Vectastain ABC Kit, Vector Laboratories) with use of the indicated primary antibodies. The samples were counterstained with hematoxylin.

The study was conducted according to the guidelines for animal experiments approved by the ethics committee at our institution.

Statistical analysis. Values are means ± SE. An unpaired t-test was used to compare the mean value between two groups. ANOVA with Scheffé's F procedure for post hoc analysis was used for comparison among three or more groups. P < 0.05 was considered statistically significant. AQ: 3

RESULTS

Role of adiponectin receptors in inhibitory effects of adiponectin on ET-1-induced hypertrophy of cultured cardiomyocytes. Compared with cultured cardiomyocytes treated with ET-1 alone, recombinant full-length adiponectin dose dependently suppressed the ET-1-induced increase in cell surface area and the cellular incorporation of [^{3}H]leucine (Figs. 1, *A* and *B*, and 2). Full-length adiponectin also inhibited the ANG II- or IGF-I-induced increase in cell surface area (Fig. 1*C*). Transfection of siRNA specific for AdipoR1 and AdipoR2 reversed the suppressive effect of full-length adiponectin on ET-1-induced cellular hypertrophy in cultured cardiomyocytes (Figs. 2 and 3, *A* and *B*), in parallel with suppression of AdipoR1 and AdipoR2 protein expression levels (Fig. 4, *A* and *B*). Also, siRNA for AdipoR1 or AdipoR2 reversed the inhibitory effect of full-length adiponectin on the ANG II- or IGF-I-induced cellular hypertrophy (data not shown). The effects of globular adiponectin were similar to those of full-length adiponectin, and siRNA for AdipoR1, but not AdipoR2,

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reversed the actions of globular adiponectin (Fig. 3*C*). Neither siRNA for AdipoR1 nor siRNA for AdipoR2 in the absence of adiponectin changed ET-1-induced cellular hypertrophy (data not shown). AQ: 4

Adiponectin induced AMPK phosphorylation and inhibited ET-1-induced ERK1/2 phosphorylation, which was also reversible by transfection of siRNA for AdipoR1 or AdipoR2 in cultured cardiomyocytes (Fig. 5, *A* and *B*). Transfection of siRNA for AMPKα_2 reduced the inhibitory effect of adiponectin on ET-1-induced cellular incorporation of [^{3}H]leucine (Fig. 3*D*) and ERK phosphorylation (Fig. 5*B*), in parallel with suppression of AMPKα_2 protein expression levels (Fig. 4*C*). Adiponectin induced ACC phosphorylation, which was also reversed by AMPKα_2 siRNA (Fig. 5*C*). F5

Effects of AICAR on ET-1-induced cellular hypertrophy and ERK phosphorylation. AICAR dose dependently inhibited the ET-1-induced increase in cell surface area of the cultured cardiomyocytes (Fig. 1*D*). AICAR inhibited ERK1/2 phosphorylation induced by ET-1 treatment but did not affect ERK1/2 phosphorylation at baseline (Fig. 1*E*).

Expression of adiponectin and its receptors in normal and infarcted hearts in animal models. Protein expression levels of AdipoR1, AdipoR2, and adiponectin in the left ventricle were similar in magnitude to those in skeletal muscle in mice (Fig. 6, *E–H*); however, mRNA levels of AdipoR1 and AdipoR2 were higher in the ventricle than in skeletal muscle (Fig. 6, *A* and *B*). The mRNA expression level of AdipoR1 was higher than that of AdipoR2 in the left ventricle (Fig. 6*D*). Compared with the normal left ventricle, expression levels of AdipoR1 mRNA and protein were decreased in the remote, as well as the infarcted, area 2 wk after myocardial infarction in mice (Fig. 7). Expression levels of AdipoR2 mRNA and protein were decreased in the infarcted area. AdipoR2 expression levels had a tendency to decrease in the remote area, but the change was not significant (Fig. 7). F6 F7

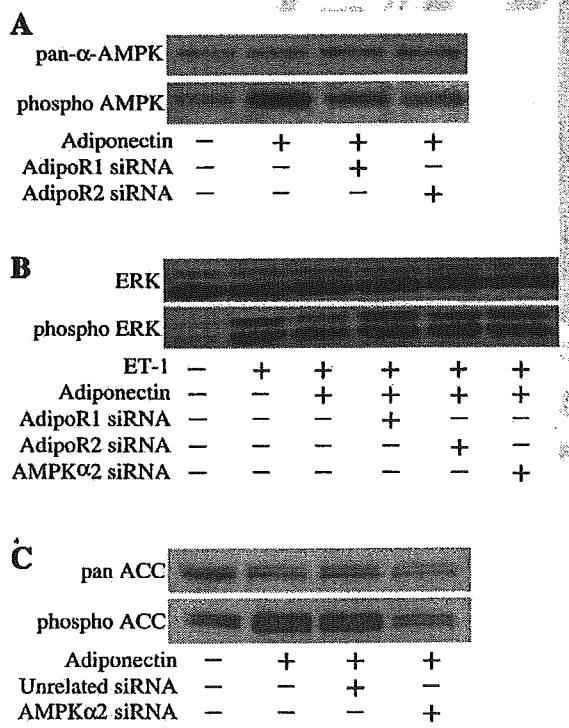

Fig. 5. Effects of suppression of AdipoR1, AdipoR2, or AMPKα_2 by siRNA on actions of adiponectin on phosphorylation of AMPK and acetyl CoA-carboxylase (ACC)- and ET-1-induced ERK phosphorylation. After transfection of each siRNA, cultured cardiomyocytes were treated for 30 min with full-length adiponectin (30 μg/ml), and cell lysates were assayed for immunoblot analysis of AMPK (*A*) and ACC (*C*) phosphorylation. Cells treated with adiponectin were additionally incubated with ET-1 (100 nmol/l) for 5 min, and treated cell lysates were assayed for immunoblot analysis of ERK1/2 phosphorylation (*B*). Results represent 3 independent experiments.

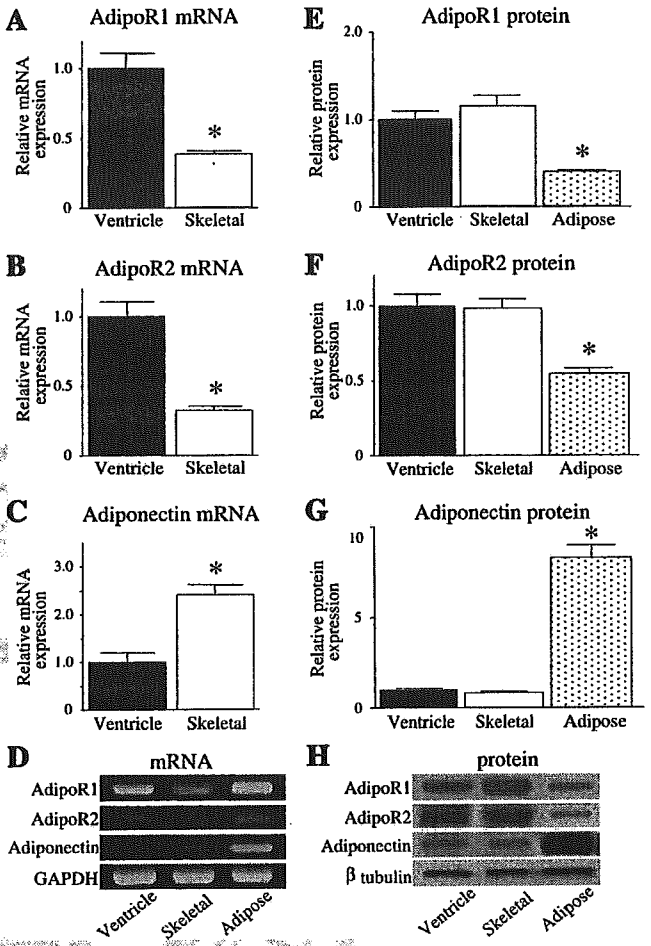

Fig. 6. mRNA and protein expression of AdipoR1, AdipoR2, and adiponectin in left ventricle, skeletal muscle, and adipose tissue in normal mouse. Total RNA (0.1 μg) was subjected to quantitative real-time PCR analysis with primers for AdipoR1 (*A*), AdipoR2 (*B*), and adiponectin (*C*). mRNA expression levels were normalized to GAPDH mRNA expression and expressed relative to left ventricle (= 1). Values are means ± SE (n = 6). *$P < 0.05$ vs. ventricle. *D*: agarose gel electrophoresis of amplified PCR products at 25 cycles from 0.1 μg of total RNA from left ventricle, skeletal muscle, and adipose tissue of normal mouse. Tissue homogenates (15 μg of protein) from left ventricle, skeletal muscle, and adipose tissue of normal mouse were subjected to immunoblot analysis with antibodies against AdipoR1 (*E*), AdipoR2 (*F*), and adiponectin (*G*). Intensity of β-tubulin band was used as a loading control between samples. Protein levels are expressed relative to left ventricle (= 1). Values are means ± SE (n = 6). *$P < 0.05$ vs. ventricle. *H*: representative immunoblots.

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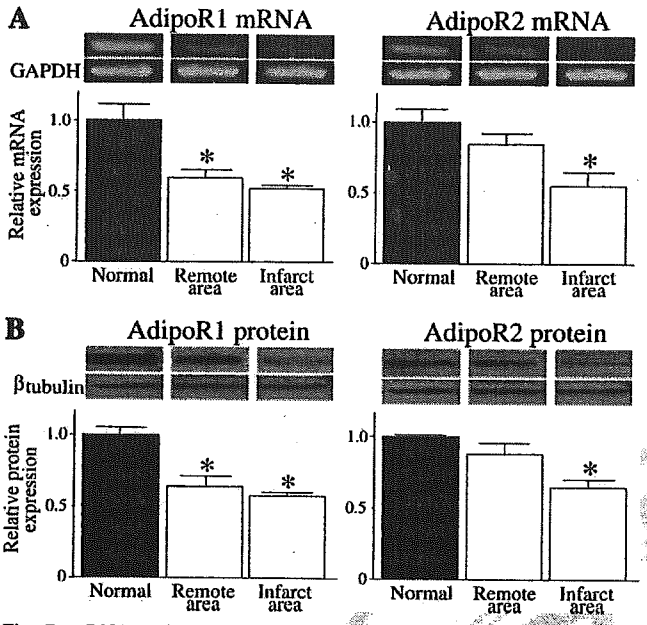

Fig. 7. mRNA and protein expression of AdipoR1 and AdipoR2 in infarcted and normal left ventricles in mouse. *A*: real-time quantitative PCR analysis of AdipoR1 and AdipoR2 mRNA expression in remote and infarcted areas of postinfarction ventricle and in normal ventricle in mouse. Levels of mRNA expression were normalized to GAPDH mRNA expression and expressed relative to normal ventricle (= 1). Values are means ± SE (n = 6). *P < 0.05 vs. normal. *Insets*: agarose gel electrophoresis of amplified PCR products from 0.1 μg of total RNA (AdipoR1 and GAPDH at 25 cycles and AdipoR2 at 30 cycles). *B*: immunoblot analysis with antibodies against AdipoR1 and AdipoR2 protein expression in remote and infarcted areas and in normal mouse ventricle. Intensity of β-tubulin band was used as a loading control between samples. Protein levels are expressed relative to normal ventricle (= 1). Values are means ± SE (n = 6). *P < 0.05 vs. normal.

Immunohistochemical staining showed that AdipoR1 and AdipoR2 were expressed mainly in myocytes of the left ventricle (Fig. 8). However, both receptors were weakly expressed in fibrous tissue of the infarcted myocardium.

Similar data regarding expression levels of AdipoR1 and AdipoR2 mRNA and protein in normal and infarcted hearts were obtained from rats (data not shown).

Effects of neurohumoral factors on mRNA expression levels of adiponectin receptors in cultured cardiomyocytes. Because TNF-α, ANG II, and norepinephrine, as well as ET-1, have been reported to play a possible role in the pathogenesis of postinfarct ventricular remodeling (4, 8, 18, 19), experiments were performed to determine the effect of these neurohumoral factors on mRNA expression levels of AdipoR1 and AdipoR2 in cultured cardiomyocytes. TNF-α and norepinephrine significantly inhibited mRNA expression levels of AdipoR1 and AdipoR2 in cultured cardiomyocytes (Fig. 9).

DISCUSSION

Using siRNAs specific for AdipoR1 and AdipoR2, we have shown that AdipoR1 and AdipoR2 mediated the suppressive effects of full-length adiponectin on ET-1-induced cardiomyocyte hypertrophy. AdipoR1 and AdipoR2 were expressed in the left ventricle and skeletal muscle to a similar extent. Furthermore, AdipoR1 and AdipoR2 expression levels were decreased in the infarcted area of the left ventricle. Also, AdipoR1 expression levels were significantly decreased in the remote area of the left ventricle. AdipoR2 expression levels had a tendency to decrease in the remote area, but the change was not significant. ET-1 has previously been shown to contribute to cardiomyocyte hypertrophy, leading to heart failure after myocardial infarction (8, 23). Therefore, the present results indicate that the myocardial expression of AdipoR1 and AdipoR2 might play a role in the regulation of cardiomyocyte hypertrophy after myocardial infarction in the remote, as well as the infarcted, area.

It has been previously shown that AMPK is involved in the signaling pathway for the metabolic effects of adiponectin (22). Furthermore, activation of AMPK was shown to suppress ERK phosphorylation, which leads to cardiac hypertrophy under pressure overloading (15). The present study showed that adiponectin induced AMPK phosphorylation in association with suppression of ET-1-induced ERK phosphorylation in cultured cardiomyocytes. Furthermore, siRNA for AMPK also suppressed the inhibitory effects of adiponectin on ET-1-induced cellular hypertrophy. Taken together, the inhibitory effects of adiponectin on ET-1-induced cellular hypertrophy may be at least partly mediated via the AMPK-ERK pathway in cultured cardiomyocytes. In support of this notion, the present study also showed that AICAR, a specific stimulator of AMPK, mimicked the results obtained with adiponectin. Furthermore, AMPK activation is known to stimulate fatty acid oxidation, which may lead to inhibition of cardiomyocyte hypertrophy (1, 6, 14, 15, 22). The present study also showed that adiponectin induced phosphorylation of ACC, an important regulator of fatty acid oxidation, through AMPK. Thus it is also possible that adiponectin might influence myocardial energy substrate utilization, including glucose uptake and fatty acid oxidation through AMPK, and, thereby, block hypertrophic growth and, in addition, the suppressive effect of AMPK on ERK phosphorylation. The present study also showed that adiponectin suppressed the hypertrophic response of cultured cardiomyocytes to IGF-I, which stimulates the IGF-I receptor, a tyrosine kinase receptor distinct from G protein-coupled receptors. It has been reported that postreceptor signaling cascades of IGF-I share the ERK pathway to cardiac hypertrophy (7). It remains to be determined whether adiponectin may uniformly suppress cardiac hypertrophy induced by stimulations converging to a common pathway with ERK.

Although adiponectin is produced in the heart, its expression in the myocardium was extremely low compared with that in adipose tissue. Therefore, circulating adiponectin, rather than adiponectin produced locally in the myocardium, seems to act as the predominant ligand for myocardial adiponectin receptors; however, adiponectin produced in the myocardium may function in an autocrine or paracrine manner. The biological activities of adiponectin have been shown to depend on the structure and the oligomeric state (10, 20). Adiponectin in human or mouse serum formed trimers, hexamers, and high-molecular-weight species (10, 20). It is not clear whether these

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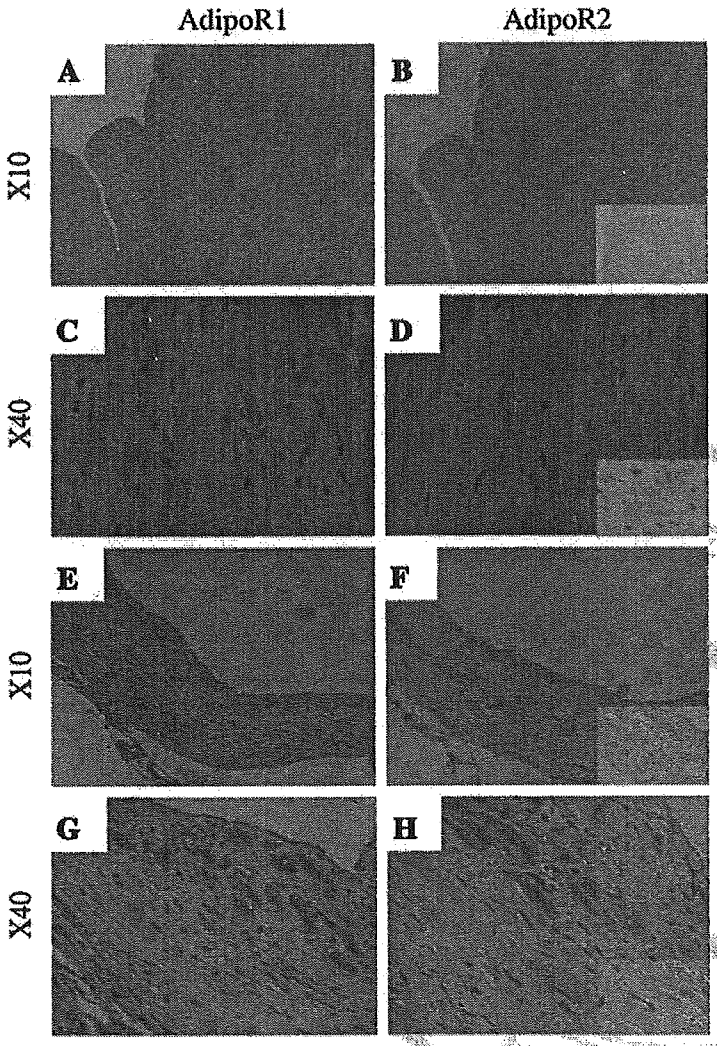


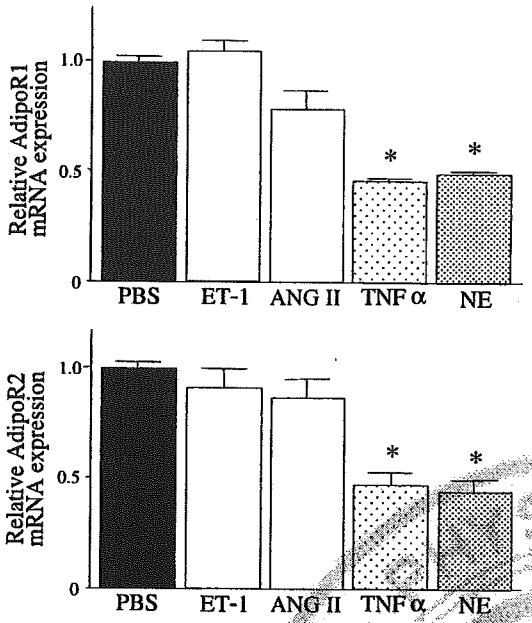
COLOR

Fig. 8. Immunohistochemical staining of mouse left ventricle with antibodies to AdipoR1 and AdipoR2. Immunoreactivity (alkaline phosphatase-linked, red) of AdipoR1 and AdipoR2. *Insets*: negative control with omission of primary antibody. *A–D*: normal ventricle. *E–H*: infarcted ventricle.

oligomers may have different affinities for AdipoR1 and AdipoR2, leading to different biological actions of adiponectin in assays in vitro. The present study using siRNA for AdipoR1 and AdipoR2 showed that both receptors were involved in the effects of full-length adiponectin, whereas AdipoR1, but not AdipoR2, mediated the effects of globular adiponectin. The different roles of AdipoR1 and AdipoR2 may be explained by the different affinity of these receptors for full-length and globular adiponectin, as previously reported (9, 21).

The precise regulatory mechanisms for the myocardial expression of AdipoR1 and AdipoR2 remain undetermined, but the present study showed that AdipoR1 and AdipoR2 expression was suppressed by TNF-α and norepinephrine, which importantly participate in the pathogenesis of myocardial remodeling (4, 18). This finding is reminiscent of counteractions between adiponectin and TNF-α on insulin sensitivity in adipocytes (5, 9, 12). The present immunohistochemical study showed that the adiponectin receptors were expressed mainly in myocytes and that they were weakly expressed in fibrous tissue of the infarcted myocardium. Therefore, a loss of cardiomyocytes may contribute to a decrease in mRNA and protein expression levels of the adiponectin receptors in the infarcted myocardium. However, it is possible that TNF-α and norepinephrine may participate in the decrease in expression of the adiponectin receptors, especially AdipoR1, in the remote myocardium of the infarcted heart. It remains to be determined whether expression of adiponectin receptors per cardiomyocyte is decreased in the surviving myocardium in the infracted area.

In conclusion, AdipoR1 and AdipoR2 mediate the inhibitory effects of adiponectin on ET-1-induced cardiomyocyte hypertrophy, and AMPK is involved in signal transduction through these receptors. The present study suggests that the myocardial expression of AdipoR1 and AdipoR2 might play a role in the pathogenesis of ET-1-related cardiomyocyte hypertrophy and subsequent heart failure after myocardial infarction. Furthermore, this study may provide a clue regarding mechanisms of cardiac metabolic disorder in ischemic heart disease.



AQ: 9 Fig. 9. Effects of ET-1, ANG II, TNF-α, and norepinephrine (NE) on mRNA expression of AdipoR1 and AdipoR2 in cultured cardiomyocytes. AdipoR1 and AdipoR2 mRNA expression levels were measured in cultured cardiomyocytes treated for 8 h with ET-1 (100 nmol/l), ANG II (100 nmol/l), TNF-α (10 ng/ml), norepinephrine (100 nmol/l), or vehicle (PBS). Values are means ± SE ($n = 6$). *$P < 0.05$ vs. PBS.

GRANTS

This study was supported by Grants-in-Aid 2-15390244, for Priority Areas and 15012222 for Medical Genome Science from the Ministry of Education, Culture, Sports, Science, and Technology and Health and Labor Sciences Research Grant for Comprehensive Research on Aging and Health H15-Choju-
AQ: 6 012 (Tokyo, Japan).

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Triglycerides and Remnant Particles As Risk Factors for Coronary Artery Disease

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Current Atherosclerosis Reports 2006, 8:xx–xx
Current Science Inc. ISSN 1523-3804

Coronary artery disease (CAD) is the largest cause of morbidity and mortality in the world. A relationship between CAD and elevated levels of low-density lipoprotein cholesterol has been established. However, risk assessment limited to low-density lipoprotein fails to identify a significant portion of patients at risk for CAD. Remnant lipoproteins, derived from very low-density-lipoprotein and chylomicrons, have been considered atherogenic. Recently, a simple and reliable immunoaffinity separation method for the isolation of remnant-like particles (RLP) has been developed. It has been shown that RLP cholesterol levels are significantly correlated with CAD, and thus cellular mechanisms have been determined by which RLP cholesterol causes progression of atherosclerosis. Measurement of RLP cholesterol is useful for the assessment of risk and the evaluation of therapy in patients at risk for CAD.

Introduction

Coronary artery disease (CAD) is the largest cause of morbidity and mortality in the world. A relationship between elevated levels of cholesterol and CAD has been established in many clinical reports. The National Cholesterol Education Program guidelines for the treatment of hypercholesterolemia in adults identify low-density lipoprotein (LDL) as the primary target of therapy [1•]. However, risk assessment limited to LDL cholesterol fails to capture a significant portion of patients at risk for CAD, and a significant number of patients effectively treated for elevated LDL cholesterol still experience progression of CAD. Thus, risk assessment using other lipoproteins such as triglycerides (TGs), and high-density lipoprotein (HDL) cholesterol is important, and elevated TGs and HDL cholesterol are additional targets of therapy [2]. Based on the combined data from prospective studies, Hokanson and Austin [3] reported that elevated TGs were a risk factor for cardiovascular disease for in both men and woman in the general population, independent of HDL cholesterol levels. Moreover, Austin et al. [4] performed a meta-analysis that showed that high TG levels were predictive of CAD risk in patients with familial combined hyperlipidemia. Therefore, the importance of TG levels has been recognized in the assessment of risk for CAD.

There is considerable inter-individual and intra-individual variation in TG levels, and circulating triglyceride-rich lipoproteins (TRLs) are highly heterogeneous in size, density, and composition and may consist of intestinally derived apolipoprotein (apo) B-48, which contains chylomicrons and chylomicron remnants, in addition to liver-derived apoB-100, which contain very low-density lipoprotein (VLDL) and its remnants [5]. TRLs comprise a great variety of nascent and metabolically modified lipoprotein particles differing in size, density, and lipid and apolipoprotein composition. The concentrations of these particles have been evaluated with a number of different methods based on their density, size, charge, specific lipid components, apolipoprotein composition, or immunoaffinity. However, these methods are limited for routine analysis in clinical laboratories because of their complexity and

cost. A novel immunoseparation method for remnant lipoproteins was recently developed by Nakajima et al. [6]. This method uses a monoclonal antibody to human apoB-100 that recognizes an epitope near B-51 to remove almost all LDL and most VLDL particles containing apoB-100, together with a monoclonal antibody to apoA-I to remove almost all HDL particles. The particles that remain unbound to these antibodies are principally all chylomicron remnants and a fraction of VLDL particles, both enriched in apoE, a characteristic of remnant lipoproteins termed remnant-like particles (RLP). The monoclonal antibodies are conjugated to Sepharose 4B to facilitate the separation of bound lipoproteins such as LDL and HDL from the RLP fraction. This immunoseparation method for isolating RLP cholesterol has been shown to be both simple and reliable and, therefore, useful for assessing and monitoring the risk of CAD [7]. The present review article summarizes our knowledge about recent clinical and biochemical evidence on the role of RLP cholesterol as a risk for CAD.

Atherogenesis and RLP Cholesterol

It has been established that elevated levels of RLP cholesterol are associated with endothelial dysfunction and atherosclerosis. Endothelial dysfunction is known to be an early event in atherosclerotic development and an important contributor to the pathogenesis of CAD. It was reported that remnant lipoproteins had a significant and independent correlation with abnormal vasomotor reactivity in human coronary arteries [8]. These results suggested that an increase in remnant levels may cause a decrease in coronary nitric oxide (NO) bioactivity, leading to impairment of endothelium-dependent dilatation in coronary arteries. Oxidative stress is a common feature of various coronary risk factors for atherosclerosis. Remnant lipoproteins may be oxidatively modified in the arterial intima and cause an increase in the susceptibility of the coronary endothelium to oxidative stress, which may play a role in the genesis of coronary endothelial dysfunction in subjects with high remnant lipoprotein levels. Furthermore, RLP upregulates endothelial expression of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are responsible for monocyte recruitment into the arterial walls. RLP also upregulates tissue factor, which is essential for thrombotic events, and this occurs at the same range of RLP concentrations that is in peripheral plasma in patients with CAD. In addition, treatment of subjects with elevated plasma levels of RLP cholesterol for 4 weeks with α-tocopherol prevented the rise in plasma levels of soluble ICAM-1 and soluble VCAM-1. Thus, high plasma levels of RLP may have an important role in development of atherosclerosis and thrombotic events through endothelial upregulation of these proatherothrombogenic molecules, partly through a redox-sensitive mechanism [9]. Incubation of RLP with human umbilical vein endothelial cells and a mononuclear cell fraction in a flow-conditioned model resulted in enhanced expression of CD11a, CD18, CD49d, and interleukin-1 β, which indicates a role of remnant lipoproteins in the initiation of vascular inflammation. Thus, RLP cholesterol plays an important role in the pathogenesis of the atherosclerotic process (Fig. 1).

Clinical Features of RLP Cholesterol

Plasma levels of RLP cholesterol in healthy normolipidemic white patients typically range from 6.2 to 9.3 mg/dL. Plasma RLP cholesterol levels increase with age in both male and female patients [10], especially in postmenopausal women. Many studies have indicated that RLP cholesterol has a significant correlation with CAD. We [11] have shown that higher levels of RLP cholesterol in fasting serum predicted the development of clinical coronary events in patients with CAD independently of other risk factors. The recurrence rate of coronary events was significantly higher in patients with RLP cholesterol in the 75th percentile ($\geq$ 5.1 mg/dL) or higher than in patients with RLP cholesterol in the 50th percentile (3.3 mg/dL) lower (Fig. 2).

A number of studies have investigated the contribution of RLP cholesterol to the atherogenic lipoprotein profile in type 2 diabetes mellitus (DM). Microangiopathy and macroangiopthy are common complications of type 2 DM. Fukushima et al. [12••] have shown that RLP cholesterol and HbA_{1c} levels were risk factors for CAD in patients with type 2 DM using multiple logistic regression analysis. Furthermore, the prospective component of this study found that increased levels of RLP cholesterol were a better predictor than high HbA_{1c} levels for the development of clinical coronary events. These results indicate that high levels of RLP cholesterol play a crucial role in the pathogenesis of macroangiopathy in type 2 DM [12••]. Thus, measurement of RLP cholesterol is also useful for the assessment of CAD risk in diabetic patients.

Metabolic Syndrome and RLP Cholesterol

Metabolic syndrome is a clustering of atherosclerotic metabolic abnormalities characterized by insulin resistance, visceral adiposity, high TGs, and low HDL cholesterol. This syndrome is highly prevalent and strongly associated with CAD [13•]. Multiple metabolic disorders contribute to the pathogenesis of this syndrome, and these metabolic disorders are intimately linked with each other. Dyslipidemia, characterized by elevated TG levels and low HDL levels, is a hallmark of metabolic syndrome. High levels of RLP cholesterol were the strongest risk factor for CAD and impaired flow-mediated dilation of the brachial artery in patients with metabolic syndrome [14••]. The predictive value of RLP cholesterol was independent of other the co-variates, including the components of metabolic syndrome and serum levels of proinflammatory markers. Increased flux of free fatty acids from the periphery to the liver might increase hepatic production and secretion of triglyceride-rich VLDL, leading to an increase in circulating levels of remnant lipoproteins in patients with metabolic syndrome. However, the causes of remnant lipoproteinemia in the metabolic syndrome are multifunctional and linked with each other and not simply a function of increased free fatty acid flux

to the liver. For example, a proinflammatory state intimately connects with dyslipidemia in the metabolic syndrome. Elevated levels of tumor necrosis factor-a and interleukin-6, which are independent risk factors for CAD in patients with metabolic syndrome, are known to increase TG levels, which could contribute to remnant lipoproteinemia. Furthermore, we [14••] demonstrated that high levels of high-sensitivity C-reactive protein (hsCRP) were also an independent risk factor for endothelial vasomotor dysfunction and CAD. When we categorized patients according to their RLP cholesterol and hsCRP levels, higher levels of RLP cholesterol and hsCRP were additive in their effect on the risk of endothelial vasomotor dysfunction and CAD in patients with metabolic syndrome (Fig. 3) [14••]. Taken together, these results are compatible with the concept that chronic subclinical inflammation is an important factor in the pathogenesis of metabolic syndrome. High RLP cholesterol levels could be a distinct pathophysiologic feature of metabolic syndrome, and thus measurement of RLP cholesterol may be useful for identification of high-risk patients with metabolic syndrome.

Lipid-lowering Medications for RLP Cholesterol

Two classes of lipid-lowering agents, statins and fibrates, have played a prominent role in such trials. Statins are known to induce LDL receptor gene expression, thereby increasing the number of LDL receptors, resulting in subsequent inhibition of cholesterol synthesis. Because the LDL receptor is also important for removal of remnant particles, statins can also improve RLP clearance, leading to a decrease in TG levels. However, statin treatment has not been associated consistently with a reduction in plasma RLP cholesterol [15•,16]. Additional intervention studies in distinct patient groups are required to compare the effect of different statins on plasma RLP cholesterol levels.

Fibrates are agonists of peroxisome proliferator activated receptor α. Fibrates primarily lower TG levels by influencing lipoprotein lipase and apoC- I I I gene expression. Fibrates also decrease the availability of fatty acids for TG synthesis and may consequently influence VLDL secretion [17], thereby influencing cholesterol levels as a secondary effect. RLP cholesterol levels are strongly correlated with TG levels and, therefore, it is predictable that fibrate treatment significantly decreases RLP cholesterol levels.

Conclusions

Measurement of RLP cholesterol is useful for the assessment of risk and therapeutic effects in patients at risk of CAD.

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Papers of particular interest, published recently,
have been highlighted as:

- • Of importance
- •• Of major importance

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Figure 1. Cellular mechanisms of remnant-like particles (RLP) for atherogenesis. ICAM–intracellular adhesion molecule; NF-kB–nuclear factor-kB; NO–nitric oxide; NOS–nitric oxide synthase; SMC–smooth muscle cell; VCAM–vascular cell adhesion molecule.

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Figure 2. Kaplan-Meier curves comparing the probability of developing coronary events according to remnant-like particle cholesterol levels during the follow-up period (≤ 36 months after enrollment) in 135 patients with coronary artery disease (n = 39, 40, and 56 in the highest [> 5.1 mg/dL], middle [3.3 ≤ 5.1 mg/dL], and lowest [≤ 3.3 mg/dL] tertiles, respectively. (*Adapted from* McNamara et al. [10].)

Figure 3. **A**, Incremental effect on odds ratio for coronary artery disease (CAD) of the combination of elevated levels of remnant-like particle (RLP) cholesterol and high-sensitivity C-reactive protein (hsCRP). **B**, Incremental effect on flow-mediated dilation of the combination of elevated levels of RLP cholesterol and hsCRP. (*Adapted from* Grundy et al. [13•].)

Dr. Kugiyama,

The figures will be sent later via fax after they have been prepared by our Art Department.

European Heart Journal (2006) 27, 817–823
doi:10.1093/eurheartj/ehi746

Elevated levels of remnant lipoproteins are associated with plasma platelet microparticles in patients with type-2 diabetes mellitus without obstructive coronary artery disease

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Received 4 January 2005; revised 21 October 2005; accepted 5 January 2006; online publish-ahead-of-print 24 January 2006

KEYWORDS
Remnant lipoprotein;
Diabetes mellitus;
Platelet-derived microparticles

Aims Platelets participate in the pathogenesis of arterial thrombosis and it has been demonstrated that enhanced platelet activation occurs in patients with diabetes mellitus (DM). Dyslipidaemia is a common feature of diabetes. We investigated the association between certain lipid fractions and plasma platelet-derived microparticle (PMP) levels in patients with type-2 DM.

Methods and results We measured fasting serum levels of remnant-like lipoprotein particles-cholesterol (RLP-cholesterol) and assessed *in vivo* platelet activation by quantifying the number of PMP in the plasma detected as CD42b-positive microparticles by flow cytometry in Japanese type-2 DM patients without obstructive coronary artery disease who were more slender when compared with Western diabetic patients. The levels of total cholesterol, triglycerides, RLP-cholesterol, and plasma glucose were significantly higher in patients with type-2 DM ($n = 105$) than in non-diabetic patients ($n = 92$). The plasma levels of PMP were elevated significantly in type-2 DM patients when compared with non-diabetic control subjects [$7.41(5.39–10.50) \times 10^6$ vs. $3.44(2.43–4.41) \times 10^6$, $P < 0.001$]. We found that RLP-cholesterol levels were the best predictor of PMP in multivariable linear regression analyses ($\beta = 0.375$, $P < 0.001$). Lipid-lowering medication with bezafibrate successfully reduced levels of both RLP-cholesterol and PMP in patients with type-2 DM ($P < 0.05$).

Conclusions RLP-cholesterol and platelet microparticles are both elevated in type-2 DM patients when compared with controls. RLP-cholesterol is the primary and only predictor of platelet microparticles in the multivariable analysis, which include several standard atherosclerosis risk factors. This suggested that reducing elevated RLP-cholesterol with lipid-lowering therapy may be an effective strategy to prevent thrombogenic vascular complications in type-2 DM.

Type-2 diabetes mellitus (DM) is associated with frequent atherothrombogenic complications and patients with the disorder have a two- to four-fold increased risk of developing coronary artery disease (CAD) compared with normal subjects. In fact, the risk of a coronary event is as high in diabetic patients without a previous myocardial infarction (MI) as it is in non-diabetic patients with a previous MI.[1] Therefore, primary prevention of cardiovascular events in diabetic patients without previous CAD is very important.[2] Dyslipidaemia is a common feature of diabetes and is known to increase the mortality of patients with diabetes and CAD.[3] Several large clinical trials have demonstrated the benefit of lipid-lowering therapy in patients with DM, as in all these trials, a reduction in LDL-cholesterol levels was associated with a decrease in the prevalence of cardiovascular events.[2,4,5]

Serum levels of remnant-like lipoproteins cholesterol (RPL-cholesterol) are increased in patients with DM.[6,7] Using an immunoseparation method for measuring RLP-cholesterol, recent clinical studies have demonstrated that these particles are closely associated with atherosclerosis.[6,8–10] We have also shown that there is a relationship between RLP-cholesterol and coronary instability in patients with CAD,[10] and also with increased gene expression of atherothrombogenic molecules.[11]

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Activated platelets participate in the pathogenesis of arterial thrombosis[12–14] and atherosclerosis.[15] It is well documented that patients with DM have enhanced platelet activation,[14] and platelet activation is associated with increased thrombogenic vascular complications in these patients. Several *in vitro* studies have shown that triglyceride-rich very-low density lipoprotein (VLDL) causes platelet activation by binding to the CD36 receptor on platelets,[16] and RPL fraction may also directly activate human platelets.[17–19] However, the involvement of lipid fractions in the enhanced thrombogenicity in patients with DM remains uncertain.

Previous reports have defined circulating platelet microparticles (PMPs) as particles <1.5 μm in diameter that are released from platelets into the extra-cellular space in response to platelet activation.[20] Several studies have shown that PMPs assessed by flow cytometry is a useful marker for evaluating platelet activation.[20–22]

In this study, we measured serum levels of lipid parameters including RLP-cholesterol and plasma levels of PMPs in order to test the hypothesis that RLP-cholesterol may contribute to platelet activation in diabetic patients without CAD.

Methods

Clinical study population

This study involved consecutive enrolment of Japanese patients who had angina-like chest symptoms or ECG abnormalities who underwent elective and diagnostic cardiac catheterization in Kumamoto University Hospital. On the basis of the coronary angiographical evaluations, we enrolled 236 patients without obstructive CAD (≤10% stenosis in coronary arteries) or peripheral artery disease (ankle brachial pressure index ≥1.0) to undergo initial assessments for inclusion into the study. Thirty-nine patients with unstable conditions such as severe valvular diseases,[5] acute infection,[2] untreated malignant disease,[1] active autoimmune disease,[1] and severe congestive heart failure,[7] or those taking any lipid-lowering medications,[11] or anti-platelet drugs[12] were excluded after the initial assessments for study. We finally enrolled 197 patients without obstructive CAD in the study and then separated this population into two groups; a type-2 diabetes group ($n = 105$) and a non-diabetic group as control ($n = 92$). Type-2 DM was diagnosed using WHO criteria. Informed consent was obtained from all patients prior to the study and this study was carried out in accordance with the guidelines approved by the Ethics Committee at our institution.

Measurement of lipoproteins and other biochemical parameters

Measurement of all the parameters with the exception of RLP-cholesterol was carried out at our hospital laboratory. RLP-cholesterol was measured as described in our earlier report.[10] The arbitrary cut-off point defining high levels of RLP-cholesterol was set at 4.6 mg/dL, a value that corresponded to the median in patients with DM. We also measured plasma levels of plasminogen activator inhibitor-1 (PAI-1), fibrinogen, and homocysteine in patients with DM.

Measurement of circulating plasma levels of PMPs

Blood samples were drawn by venipuncture into vacutainer tubes containing sodium citrate after a 12-h overnight fast, prior to any mechanical intervention. The blood samples were assayed immediately after venipuncture. Platelet-rich plasma (PRP) was prepared by centrifuging whole blood at 160 *g* for 10 min. The PRP was then centrifuged at 6000 *g* for 1 min to obtain platelet-poor plasma (PPP). For the PMP assay, 50 μL of PPP in TruCount tubes (Becton Dickinson, NJ, USA) was incubated with CD42b-phycoerythrin (PE) (BD Pharmingen, San Diego, USA) for 30 min. Then, 1 mL of phosphate-buffered saline was added, and the samples were analysed using flow cytometry. PMPs were defined as elements with CD42b-positivity and a diameter <1.5 μm.[21,23] The absolute number of PMPs were calculated as described previously.[24] The arbitrary cut-off point that defined a high level of PMP was set at 7.26×10^6 counts/mL that corresponded to the 90th percentile of the PMP distribution in the control patients. The intra- and inter-assay variations for the PMP assay were $1.8 \pm 1.5\%$ and $3.1 \pm 1.7\%$ (mean ± SD), respectively.

Bezafibrate treatment and follow-up study

DM patients with elevated levels of both RLP-cholesterol (>4.6 mg/dL) and PMPs ($>7.26 \times 10^6$/mL) were recruited for a pharmacological intervention follow-up study. The 20 patients enrolled had not used any lipid-lowering drugs before entering the study. Informed consent was obtained from all the patients and they were then divided randomly into two groups, a control group not taking any lipid-lowering medications ($n = 10$), and a group treated with bezafibrate at a dose of 400 mg per day for 6 weeks (bezafibrate group, $n = 10$). The serum levels of lipid-parameters and plasma levels of PMPs were measured at baseline and after 6 weeks of treatment. As we were interested in the relative changes from baseline, we investigated whether the percentage change in PMPs had correlation with the percentage change in any lipid parameters.

Statistical analysis

The statistical analyses were performed with Stat View-V software (SAS Institute, NY, USA). The results of normal distributed data were expressed as mean ± SE, whereas non-normally distributed data such as triglycerides, fasting plasma glucose, haemoglobin A_1c (HbA_1c), RLP-cholesterol, PAI-1, and PMP levels were expressed as median and inter-quartile range. The frequencies for gender, smoking, and hypertension were compared between the two groups using χ^2 analysis. Comparisons between the two groups were carried out using the unpaired two-sided *t*-test for normally distributed variables (age, body mass index, total cholesterol, LDL-cholesterol, HDL-cholesterol, fibrinogen, and homocysteine) and the Mann–Whitney *U* test for non-normally distributed data (triglyceride, fasting plasma glucose, HbA_1c, RLP-cholesterol, PAI-1, and PMPs). In order to reduce the experiment-wise type I error due to multiple testing, we performed multivariable linear regression analysis using only the covariates that showed more significant association ($r > 0.35$, $P < 0.01$) in the univariate linear regression analysis. The PMP data were logarithmically transformed (log-PMP) in order to obtain a normal distribution and were then analysed using linear regression analysis. A *P*-value <0.05 was considered as statistically significant.

Results

Elevated plasma levels of PMP in patients with DM

The clinical characteristics of the patients at baseline are summarized in *Table 1*. Fasting serum levels of total-cholesterol, triglyceride, RLP-cholesterol, plasma glucose, and HbA_1c were significantly higher in patients with DM when compared with the non-DM controls. Patients with DM also had significantly lower HDL-cholesterol levels than the non-diabetic control group (*Table 1*). The levels of circulating PMPs were significantly higher in patients with DM ($n = 105$) than in the non-diabetic controls ($n = 92$) [$7.41(5.39–10.50) \times 10^6$ counts/mL vs. $3.44(2.43–4.41) \times 10^6$ counts/mL, $P < 0.001$, *Figure 1A*).

Table 1 Clinical characteristics of the subjects in the study

	Controls ($n = 92$)	DM ($n = 105$)	P-value
Male/female (n)	52/40	68/37	0.3
Age (years)	64.4 ± 1.2	62.4 ± 0.9	0.2
Body mass index (kg/m^2)	23.7 ± 0.4	23.9 ± 0.4	0.6
Hypertension (n, %)	36 (39)	54 (51)	0.1
Smoking (n, %)	23 (25)	24 (23)	0.9
Platelets (×10^3 counts/mm^2)	197.8 ± 6.4	208.2 ± 17.1	0.6
Total cholesterol (mg/dL)	189.5 ± 3.0	201.5 ± 3.9	0.02
LDL-cholesterol (mg/dL)	116.5 ± 2.7	121.1 ± 3.4	0.3
HDL-cholesterol (mg/dL)	58.4 ± 1.6	53.8 ± 1.6	0.04
Triglyceride (mg/dL)	94.5 (63.5–124.0)	119.0 (87.0–171.5)	0.003
High-sensitivity CRP (mg/dL)	0.09 ± 0.01	0.18 ± 0.04	0.09
Fasting plasma glucose (mg/dL)	95.5 (87.0–106.0)	137.0 (113.0–165.0)	<0.001
Duration of diabetes (years)	–	9.0 ± 0.8	–
Haemoglobin A_1c (%)	5.2 (5.0–5.6)	7.0 (6.3–7.8)	<0.001
RLP-cholesterol (mg/dL)	3.6 (3.0–4.8)	4.6 (3.5–6.3)	<0.001
Lipoprotein (a) (mg/dL)	17.1 ± 1.4	19.1 ± 2.2	0.2
PMPs (×10^6/mL)	3.44 (2.43–4.41)	7.41 (5.39–10.50)	<0.001
Medication therapy			
Sulfonylurea (n, %)	–	50 (48)	–
α-Glucosidase inhibitor (n, %)	–	28 (27)	–
Insulin (n, %)	–	19 (18)	–

Values are mean ± SE.
CRP, C-reactive protein;
HDL, high-density lipoprotein; LDL, low-density lipoprotein.

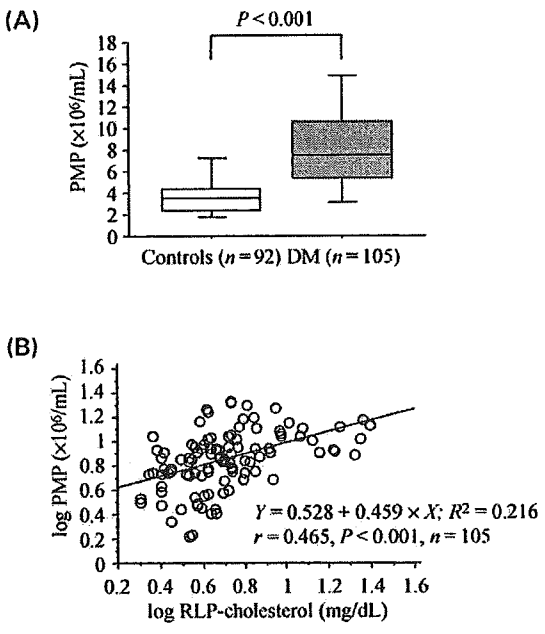

Figure 1 (*A*) A box and whisker plot showing plasma PMP levels in patients with ($n = 105$) and without DM ($n = 92$). In this plot, lines within boxes represent median values, the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively, and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (*B*) A graph demonstrating the significant correlation between RLP-cholesterol and PMP levels in patients with DM ($n = 105$) assessed using linear regression analysis.

Clinical characteristics of DM patients grouped according to RLP-cholesterol levels

The clinical and biochemical characteristics of the DM patients grouped according to high (>4.6 mg/dL) and low RLP-cholesterol levels are summarized in *Table 2*. The high RLP-cholesterol group had significantly increased levels of PMPs, body mass index, total-cholesterol, LDL-cholesterol, triglyceride, HbA_1c, PAI-1, fibrinogen, and homocysteine compared with the group with low levels of RLP-cholesterol.

RLP-cholesterol is the most significant risk factor for elevated platelet microparticles in patients with DM

In the patients with DM, univariate linear regression analysis showed that there was a significant correlation between PMP levels and serum levels of RLP-cholesterol ($r = 0.465$, $P < 0.001$), total-cholesterol ($r = 0.376$, $P < 0.001$), LDL-cholesterol ($r = 0.370$, $P < 0.001$), homocysteine ($r = 0.273$, $P < 0.03$), PAI-1 ($r = 0.261$, $P < 0.04$), and HbA_1c ($r = 0.251$, $P = 0.01$). However, HDL-cholesterol did not have correlation with PMP levels (*Table 3*). In the multivariable linear regression analysis, risk factors were found to have striking significance ($r > 0.35$, $P < 0.01$) with the univariate analysis. RLP-cholesterol was one of the risk factors that showed a significant association with elevated levels of PMPs as a marker of platelet activation in patients with DM ($\beta = 0.375$, $P < 0.001$, *Table 3*).

The effect of bezafibrate treatment on serum levels of RLP-cholesterol and plasma levels of PMP

Although there was no difference in lipid parameters and PMP levels between the two groups of DM patients at baseline, the levels of RLP-cholesterol, PMP, and triglyceride were decreased significantly in the bezafibrate group at the end of follow-up when compared with the control group. Moreover, levels of triglyceride (33%), RLP-cholesterol (45%), and PMP (53%) were significantly decreased

Table 2 The clinical characteristics of the patients with DM grouped according to RLP-cholesterol levels

	Low-RLP (≤4.6 mg/dL, $n = 50$)	High-RLP (>4.6 mg/dL, $n = 55$)	*P*-value
Male /female (*n*)	32/18	36/19	0.9
Age (years)	64.1 ± 1.2	60.9 ± 1.2	0.07
Body mass index (kg/m^2)	23.0 ± 0.5	24.8 ± 1.2	0.01
Hypertension (*n* %)	28 (52)	26 (48)	0.5
Smoking (*n* %)	9 (18)	15 (27)	0.4
Platelets (×10^3 counts/mm^2)	196.0 ± 10.8;	216.9 ± 28.0	0.6
Total cholesterol (mg/dL)	182.7 ± 4.9	217.6 ± 4.8	<0.001
LDL-cholesterol (mg/dL)	107.0 ± 4.6	133.5 ± 4.4	<0.001
HDL-cholesterol (mg/dL)	55.9 ± 2.4	51.9 ± 2.0	0.2
Triglyceride (mg/dL)	92.0 (71.5–121.5)	152.0 (112.0–221.0)	<0.001
High-sensitivity CRP (mg/dL)	0.10 ± 0.03	0.25 ± 0.07	0.2
Fasting plasma glucose (mg/dL)	125.5 (110.5–155.0)	147.5 (120.0–172.0)	0.06
Duration of diabetes (years)	9.6 ± 1.3	8.5 ± 0.9	0.5
Haemoglobin A_1c, (%)	6.8 (5.9–7.7)	7.4 (6.7–8.2)	0.006
PMPs (×10^6/mL)	5.68 (3.55–8.67)	8.58 (6.72–11.84)	<0.001
PAI-1 (ng/mL)	22.0 (13.0–32.3)	32.0 (21.5–42.5)	0.03
Fibrinogen (mg/mL)	280.8 ± 9.9	308.5 ± 13.3	0.04
Homocysteine (nmol/mL)	7.9 ± 0.4	8.7 ± 0.4	0.04
Lipoprotein (a) (mg/dL)	19.7 ± 3.4	18.7 ± 2.9	0.8
Medication.Therapy			
Sulfonylurea (*n* %)	28 (56)	22 (40)	0.1
α-Glucosidase inhibitor (*n* %)	13 (26)	15 (27)	0.9
Insulin (*n* %)	7 (14)	12 (22)	0.4

Values are mean ± SE.
PAI-1 = plasminogen activator inhibitor-1.

Table 3 Analyses between biochemical parameters and platelet microparticles in patients with DM

		95% CI	*P*-value
Univariate linear regression analysis	*r-value*		
Total-cholesterol (mg/dL)	0.376	0.19–0.51	<0.001
LDL-cholesterol (mg/dL)	0.370	0.19–0.53	<0.001
Triglyceride (mg/dL)	0.276	0.098–0.45	0.004
HDL-cholesterol (mg/dL)	−0.140	−0.34–0.37	0.2
RLP-cholesterol (mg/dL)	0.465	0.30–0.60	<0.001
Lipoprotein (a) (mg/dL)	−0.159	−0.33–0.096	0.2
Haemoglobin A_1c (%)	0.251	0.058–0.43	0.01
Fasting plasma glucose (mg/dL)	0.091	−0.11–0.28	0.4
High-sensitivity CRP (mg/dL)	0.045	−0.151–0.238	0.7
PAI-1 (ng/mL)	0.261	0.016–0.476	0.04
Fibrinogen (mg/dL)	0.196	−0.053–0.421	0.1
Homocysteine (nmol/mL)	0.273	0.03–0.487	0.03
Multivariable linear regression analyses	*β-value*		
RLP-cholesterol (mg/dL)	0.375	0.170–0.547	<0.001
LDL-cholesterol (mg/dL)	0.190	−0.214–0.742	0.2
Total-cholesterol (mg/dL)	0.05	−0.062–0.10	0.7

and HDL-cholesterol levels (12%) were significantly increased in the bezafibrate group when compared with the control group (*Table 4*). *Figure 2* shows the relationship between the percentage changes from baseline to follow-up in plasma PMP levels and RLP-cholesterol, triglyceride, total-cholesterol, and LDL-cholesterol. The percentage change in RLP-cholesterol correlated significantly with the percentage change in PMPs ($r = 0.561$, $P = 0.01$), whereas there was no significant relationship between the percentage change in PMPs and the percentage change in triglyceride ($r = 0.258$, $P = 0.2$), total-cholesterol ($r = 0.135$, $P = 0.6$), or LDL-cholesterol ($r = 0.231$, $P = 0.3$).

Discussion

This study demonstrated that patients with type-2 DM without CAD have enhanced platelet activation, assessed by quantifying the number of PMPs in the plasma.

Table 4 Baseline and follow-up data of the patients with DM in the intervention study

	Control ($n = 10$)	Bezafibrate ($n = 10$)	*P*-value
Total-cholesterol, mg/dL			
Baseline	216.6 ± 12.7	210.8 ± 7.0	0.7
Follow-up	210.0 ± 4.5	194.9 ± 8.6	0.1
Change (%)	1.1 ± 8.9	−4.7 ± 5.3	0.6
LDL-cholesterol (mg/dL)			
Baseline	129.9 ± 11.8	132.4 ± 9.1	0.9
Follow-up	130.9 ± 6.4	121.7 ± 6.8	0.3
Change (%)	16.1 ± 24.1	−1.2 ± 12.0	0.5
HDL-cholesterol (mg/dL)			
Baseline	48.2 ± 7.1	45.8 ± 3.3	0.8
Follow-up	45.4 ± 5.9	52.4 ± 5.2	0.4
Change (%)	−3.7 ± 3.1	12.3 ± 5.0	0.02
Triglyceride (mg/dL)			
Baseline	170 (134–211)	151 (135–200)	0.9
Follow-up	204 (105–221)	105 (86–120)	0.03
Change (%)	10.9 ± 16.5	−33.1 ± 6.0	0.02
RLP-cholesterol (mg/dL)			
Baseline	7.2 (6.1–9.8)	6.9 (5.8–9.4)	0.8
Follow-up	9.9 (6.6–10.9)	3.8 (3.3–4.1)	0.03
Change (%)	12.6 ± 17.7	−45.1 ± 8.4	0.008
PMPs × 10^6counts/mL			
Baseline	11.0 (8.8–13.7)	12.3 (8.9–16.4)	0.7
Follow-up	10.5 (8.7–11.8)	5.1 (4.3–8.1)	0.002
Change (%)	−2.7 ± 7.8	−53.1 ± 4.2	<0.001

Furthermore, we observed that among traditional cardiovascular risk factors and various lipid parameters, a high level of RLP-cholesterol was the only significant determinant of platelet activation, whereas pharmacological intervention with bezafibrate to decrease serum RLP-cholesterol resulted in successful reduction of PMP levels in patients with DM. Taken together, these findings indicate that remnant lipoproteinaemia may contribute partly to platelet-activation in patients with DM without obstructive CAD, and that RLP-cholesterol may therefore be a therapeutic lipid target with the potential to decrease enhanced thrombogenicity and also to prevent cardiovascular events in patients with DM.

It is well established that patients with type-2 DM develop more atherothrombogenic complications when compared with non-diabetic patients.[1] Several clinical trials have indicated that lipid-lowering therapies have an important role in the primary prevention of cardiovascular events in diabetes patients.[2,4,5] Although the possible involvement of specific lipid-fractions in these thrombogenic complications remains unclear, there is evidence that serum levels of RLP-cholesterol are elevated in patients with DM and CAD and that this lipoprotein fraction predicts future coronary events.[6,7] These findings indicate that RLP-cholesterol may play a crucial role in the pathogenesis of vascular thrombogenic events in these patients and there are several lines of evidence showing the atherogenic nature of RPL.[6,8,9]

In the present study, we assessed activation of platelets by measuring the number of CD42b-positive PMPs in the plasma using flow cytometry. CD42b is a 170 kDa two-chain membrane glycoprotein GPIb found only on platelets and megakaryocytes.[25] Previous reports have defined circulating PMPs as particles <1.5 μm in diameter that are released from platelets into the extra-cellular space in response to platelet activation.[20] Elevated levels of PMPs in the plasma have been associated with acute coronary syndrome, DM, and hypertension.[21,22,26] Given that platelet activation is associated with thrombus formation,[12,14] PMPs may therefore represent a new clinical marker for evaluating the degree of platelet activation.[20] Furthermore, PMPs play an important role in clinical diseases as they contain phospholipids and membrane proteins that have procoagulant potential and are involved in inflammatory processes.[27] Therefore, PMPs may not only be a marker of platelet activation but also a pathophysiological mediator leading to atherothrombosis.

We have shown in patients with DM that serum levels of RLP-cholesterol are associated closely with PMPs as a new marker of platelet activation. We therefore consider that high RLP-cholesterol may be linked, in part, to the initiation and progression of atherogenesis and thrombogenesis as a result of its ability to induce platelet activation in patients with type-2 DM without obstructive CAD. Although the mechanism leading to this activation is yet to be established, it has been demonstrated that RLP-cholesterol increases intracellular oxidative stress thereby causing impairment of *in vitro* endothelial-dependent vasorelaxation,[11,28] whereas other studies have shown that oxidative stress and reduction of nitric oxide (NO) induces platelet activation.[29–31] Furthermore, Englyst *et al.*[16] showed that CD36 is a receptor/transporter that binds the fatty acids of VLDL to platelets and enhances *in vitro* production of platelet thromboxane A_2. These findings therefore indicate that raised levels of RLP-cholesterol in diabetes may potentially contribute to platelet activation by increasing oxidative stress, reducing NO bioavailability, and binding lipoprotein fatty acid to the CD36 receptor. Moreover, RLP-cholesterol also directly activate human platelets.[17–19] However, the molecular mechanisms involved in the activation of platelets by RLP-cholesterol require further investigation.

Type-2 DM patients with higher RLP-cholesterol levels had a significantly higher BMI and HbA_1c levels in the present study

Several studies have reported that weight loss in obese women and metabolic control by intensive insulin treatment reduced *in vivo* platelet activation and triglyceride levels.[32–35] Thus, better glycaemic control or of weight loss might have good effects on RLP-cholesterol and PMP levels. Tenenbaum *et al.*[36] reported that bezafibrate reduces the incidence of myocardial infarction in patients with metabolic syndrome In the present study, a decrease in RPL by treatment with bezafibrate successfully reduced plasma PMP levels (*Table 4* and *Figure 2*). We therefore propose that monitoring changes in plasma PMP and serum RLP-cholesterol levels may be useful for evaluating thrombogenic disease activity in DM patients with the aim of preventing cardiovascular complications.

This study had several limitations, the first being the small size of the patient groups. The second limitation was that

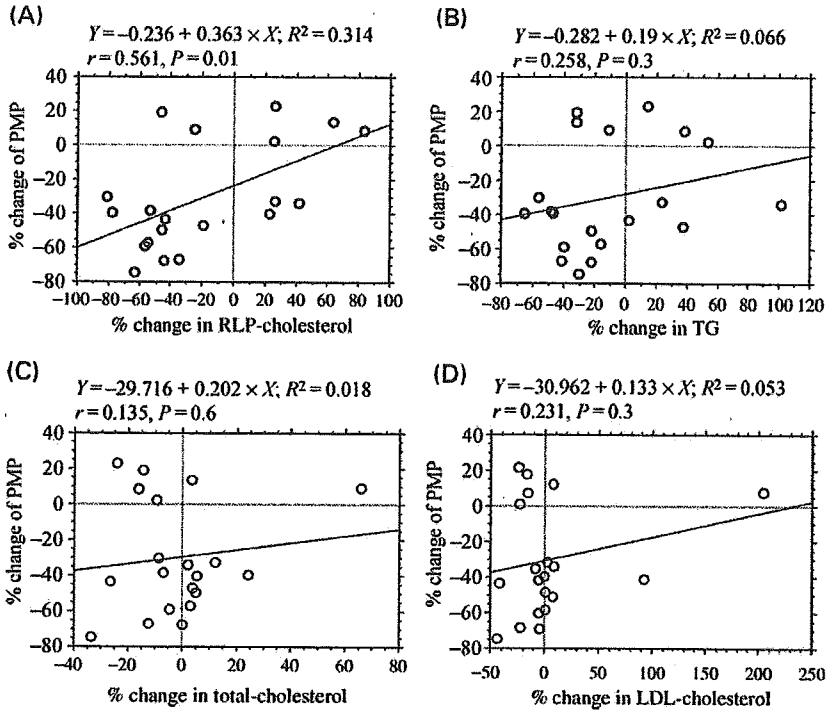

Figure 2 Relationship between the percentage changes from baseline to follow-up in plasma PMP levels and RLP-cholesterol (*A*), triglyceride (*B*), total-cholesterol (*C*), and LDL-cholesterol (*D*). The percentage change in RLP-cholesterol correlated significantly with the percentage change in PMPs. In contrast, there was no correlation between the percentage changes in plasma PMP levels and either triglyceride, total-cholesterol, or LDL-cholesterol.

Japanese diabetic patients generally have a more slender body shape when compared with Western diabetes patients. However, regardless of ethnicity it is well established that diabetes patients have an increased prevalence of cardiovascular diseases and thrombogenic complications than non-diabetic patients, indicating that the presence of DM is of primary importance in the development of these vascular disorders. We consider our results are therefore also applicable to Western DM patients who may even have a higher risk of atherothrombosis because of elevated levels of RLP-cholesterol in combination with increased BMI. The third limitation was the suppression of cardiovascular events in patients with DM treated by bezafibrate could not be verified because of the short duration of follow-up. A longitudinal prospective study of platelet activity assessed by measuring PMP levels in a large number of patients is therefore required.

In summary, our results demonstrate that platelets are activated in patients with type-2 DM without CAD and that an elevated level of RLP-cholesterol is one of the risk factors with a significant relationship with this enhanced platelet activation. Furthermore, a reduction in RLP-cholesterol by bezafibrate treatment was associated with a decrease in platelet activation. These findings imply that platelet activation induced by increased RLP-cholesterol levels may play an important role in vascular thrombogenic complications in patients with type-2 DM. Treatment of remnant lipoproteinaemia therefore has the potential not only to improve the disorder of lipoprotein metabolism but also to suppress the enhanced thrombogenicity that occurs in patients with type-2 DM.

Acknowledgements

This study was supported in part by grants-in-aid C(2)-17590753 from the Ministry of Education, Science, and Culture, Tokyo; 14C-4 and 1116004 from the Ministry of Health, Labour, and Welfare, Tokyo; The Naito Foundation; Mochida Memorial Foundation for Medical and Pharmaceutical Research; and the Suzuken Memorial Foundation, Tokyo, Smoking Research Foundation, Tokyo, Japan Heart Foundation.

Conflict of interest: none declared.

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Journal of the American College of Cardiology
© 2005 by the American College of Cardiology Foundation
Published by Elsevier Inc.

Vol. 46, No. 4, 2005
ISSN 0735-1097/05/$30.00
doi:10.1016/j.jacc.2005.04.055

Endothelial Vasomotor Dysfunction in the Brachial Artery Is Associated With Late In-Stent Coronary Restenosis

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OBJECTIVES This study examined whether endothelial dysfunction in the brachial artery might be associated with late in-stent restenosis (ISR) after percutaneous coronary intervention (PCI).

BACKGROUND Simple and noninvasive identification of late ISR might help to select patients who require further angiographic evaluation.

METHODS Endothelium-dependent flow-mediated dilation (FMD) of the brachial artery was measured before (initial FMD) and at six months (follow-up FMD) after PCI in 141 consecutive patients who had elective and successful PCI with bare metal stents in de novo lesions of native coronary arteries for symptomatic coronary artery disease. Follow-up angiography was performed at six months after PCI in all patients.

RESULTS With multivariate logistic regression analysis, the impairment (≤4.8% dilation from baseline diameter) of FMD at follow-up showed the strongest association with late ISR (defined as >50% diameter stenosis, n = 46) independently of other clinical and angiographic variables known to be associated with ISR (odds ratio 7.4, 95% confidence interval 2.8 to 19.2, $p < 0.001$), whereas the initial FMD did not have the association. The sensitivity of impaired FMD at follow-up (69%) in detecting ISR was higher than chest pain during the follow-up period (45%), with comparable specificity. The impaired FMD in combination with the chest pain increased the sensitivity to 90%.

CONCLUSIONS The impairment of FMD in the brachial artery at the time of follow-up was independently and closely associated with late ISR in native coronary arteries. The noninvasive assessment of FMD at the time of follow-up might be useful for identification of late ISR. (J Am Coll Cardiol 2005;46:648–55) © 2005 by the American College of Cardiology Foundation

In-stent restenosis (ISR), although less frequent than post-angioplasty restenosis, remains a clinical problem, because there is increasing use of coronary stents for the treatment of coronary artery disease (CAD). When chest pain develops during the follow-up period after stenting, patients might be recommended for angiographic evaluation to detect ISR or another coronary stenosis; however, several reports (1–3) have shown that approximately 50% of patients remain asymptomatic when restenosis occurs; thus, chest pain after percutaneous coronary intervention (PCI) is a poor indicator of restenosis. Therefore, a simple and noninvasive method for identifying late restenosis after PCI might help to select patients who require further angiographic evaluation.

The vascular endothelium suppresses intimal hyperplasia (4,5), an essential pathological feature of ISR (6,7). Furthermore, it has been shown that endothelial dysfunction in systemic arteries is a strong predictor of future coronary events (8,9). Thus, endothelial vasomotor dysfunction in systemic arteries in patients with coronary stenting might be associated with the development of ISR. In this study, we evaluated the usefulness of measuring endothelium-dependent dilation of the brachial artery for the identification of ISR.

METHODS

Study patients. This study included 141 consecutive patients who had elective and successful PCI of de novo lesions with bare metal stents in native coronary arteries for symptomatic CAD and follow-up coronary angiography at six months after the PCI at Yamanashi University Hospital. Patients who had acute coronary syndrome, stroke, or other serious diseases occurring during the six-month follow-up periods were excluded. Patients with congestive heart failure, left main trunk disease, and other serious systemic diseases were also excluded. This study also included 48 control subjects with angiographically normal coronary arteries and normal ventriculography. These control subjects were selected to match the age and gender of the patients

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Manuscript received January 23, 2005; revised manuscript received April 19, 2005, accepted April 25, 2005.

Abbreviations and Acronyms

CAD = coronary artery disease
ECG = electrocardiography
FMD = flow-mediated dilation
HDL-C = high-density lipoprotein cholesterol
hsCRP = high-sensitivity C-reactive protein
ISR = in-stent restenosis
MLD = minimal lumen diameter
PCI = percutaneous coronary intervention
TLR = target lesions revascularization (defined as repeat percutaneous coronary intervention of the original stented target lesions)

with PCI, and they were studied to compare endothelial vasomotor function with that of the PCI patients. The characteristics of the patients and control subjects are shown in Table 1. All patients were informed that the follow-up angiography would be required, regardless of ischemia/anginal symptoms, according to the study protocol. Written informed consent was obtained from all patients and control subjects before the study. This study was in agreement with the guidelines approved by the ethics committee at our institution.

Study protocol. Measurement of flow-mediated dilation (FMD) in the brachial artery was performed in the morning after an overnight fast in the same manner in all study patients, within three days before the PCI and within three days before the follow-up coronary angiography, at the end of the six-month follow-up period. An exercise treadmill electrocardiographic (ECG) test was performed in the morning after overnight fasting a day before the follow-up FMD. Vasodilators, including calcium blockers, angiotensin-converting enzyme inhibitors, and angiotensin receptor blockers were withdrawn 48 h before the FMD measurement and the exercise stress test. Beta-blockers were discontinued more than 12 h before the FMD measurement and the exercise stress test. Sublingual nitroglycerin was allowed to be used when ischemia/anginal symptoms were developed. All of the examinations were performed during hospital stay at Yamanashi University Hospital. The ECG was continuously and carefully monitored during the hospital stay to ensure the safety of the patients. All patients were routinely questioned for the presence or absence of chest pain during the six-month follow-up period according to Canadian Cardiovascular Society angina classification class (10) by investigators (Drs. Saito and Fujioka) without knowledge of the follow-up angiographic results. In addition, physical activity (average min/day), especially leisure-time activity, was assessed with a questionnaire at the end of the follow-up period. All of the patients received standard medical therapy during the follow-up period. Venous blood was obtained from all patients immediately before FMD measurement. High-sensitivity C-reactive protein (hsCRP) levels in the serum were assayed by rate nephelometry (Dade Behring, Marburg, Germany).

PCI. Coronary angioplasty was performed with the Judkins technique without intracoronary ultrasound scanning guidance under systemic heparinization and oral administration of aspirin and ticlopidine. The stent type and inflation pressure were chosen at the discretion of the physicians (Drs. Takano, Umetani, and Obata), who were blinded to the study protocol and the data regarding FMD. Rotablator and directional atherectomy were not performed in the stented coronary lesions in any study patients. Procedural success was defined as a residual lumen narrowing <20%. After PCI, patients received aspirin (100 mg/day) indefinitely and ticlopidine (200 mg/day) at least for four weeks. Original stented target lesions revascularization (TLR) was defined as repeated PCI or surgical bypass of the original stented lesions and was performed in the presence of ISR and any symptoms or objective signs of myocardial ischemia. Even if the symptoms or the objective signs were absent, TLR was performed in the presence of ≥75% diameter stenosis in the stented lesions.

Quantitative coronary angiography. All patients had coronary angiography before and immediately after PCI and at the planned six-month follow-up in multiple projections after intracoronary injection of 1 mg of isosobide dinitrate. Quantitative coronary angiography was conducted with the projection that revealed the highest degree of stenosis. Measurements were performed with CARDIO500 (Kontron Instruments Inc., Everett, Massachusetts) by operators (Drs. Ichigi and Mende) who were blinded to the FMD data. Lesion length, reference lumen diameter, minimal lumen diameter (MLD), stented segment length, and diameter stenosis were measured with an automated edge-detection system. Late lumen loss was defined as the difference between the post-PCI MLD and the MLD at the six-month follow-up. When a lesion was totally occluded, the lesion length was measured after opening the occlusion. In-stent restenosis was defined as >50% diameter stenosis at the stented site on the follow-up angiogram. Patients that required stents in multilesions were classified as positive for ISR, if it occurred in at least one lesion. The lesion with the greatest lumen loss was analyzed in patients with multilesions intervention.

Measurements of FMD in the brachial artery. Vasodilator responses in the brachial arteries were measured with B-mode ultrasound images with a 7.5-MHz linear array transducer (HP-5500, Phillips Corp., Tokyo, Japan), as validated in our previous studies (11,12). Measurements were performed by two observers (Drs. Nakamura and Kitta) who were blinded to the coronary angiography data. The brachial artery was scanned in the antecubital fossa in a longitudinal fashion. Optimal brachial artery images were obtained between 1 and 5 cm above the antecubital crease. This location was marked, and all subsequent images were obtained at the same location. The exact distance of the measured point of the skin surface from the antecubital crease was recorded in each subject to ensure that the same segment of the brachial artery was measured at each time

point during follow-up. The gain setting was optimized at the beginning of the study and was kept constant throughout the recording period. After baseline measurements of the diameter and flow velocity in the brachial artery, a blood pressure cuff was placed around the forearm and inflated to a pressure of 250 to 300 mm Hg for 5 min and then released. Diameter measurements during reactive hyperemia were taken 45 to 90 s after cuff deflation. Then, sublingual nitroglycerin (0.3 mg) was administered, and three min later, the measurements were repeated. Images were recorded on a super-VHS videocassette recorder (model BR-S601M, Victor Corp., Tokyo, Japan), and brachial arterial diameters were measured from the tape with ultrasonic calipers as described previously (11,12). The response of the vessel diameter to reactive hyperemia and nitroglycerin were expressed as a percentage increase in diameter from the baseline value. The diameter responses were assessed at three points along a 10-mm length of the artery, and the diameter responses were averaged. Blood flow was calculated by multiplying the velocity-time integral of the Doppler flow signal by heart rate and the vessel cross-sectional area. The increase in brachial blood flow was calculated as the maximum flow recorded in the first 15 s after cuff deflation and was expressed as a percentage increase in flow from the baseline value.

Exercise stress test. Symptom-limited treadmill exercise testing was performed with the Bruce protocol while recording a 12-lead ECG in 116 (82%) patients in the morning after overnight fasting at the end of the six-month follow-up period. The remaining 25 (18%) patients could not have the exercise stress test because of disability. A cuff blood pressure was recorded every min before and during exercise. Vasodilators and beta-blockers were discontinued more than 12 h before the exercise test. The exercise stress test was considered positive if >0.1 mV ST-segment depression occurred with or without chest pain.

Statistical analysis. Data are expressed as mean ± SD unless otherwise indicated. The mean value and frequency between two groups were compared with the Student unpaired *t* test and chi-square analysis, respectively. The mean values among three groups were compared with one-way analysis of variance, followed by a Scheffe test for post-hoc comparisons between groups. Chi-square test followed by Tukey test was used for comparing frequencies

Table 1. Comparisons of Clinical, Lesion, and Procedural Variables at Baseline Between Patients With and Without ISR

	Controls (n = 48)	With ISR (n = 46)	Without ISR (n = 95)
Clinical variables			
Age (yrs)	65 ± 12	66 ± 13	65 ± 12
Male (%)	69	66	69
Hypertension (%)	40	69*	73*
Diabetes mellitus (%)	17	32	29
Smoker (%)	23	41	34
Family history (%)	18	20	20
Total cholesterol (mg/dl)	200 ± 38	198 ± 29	200 ± 40
LDL cholesterol (mg/dl)	118 ± 32	119 ± 29	122 ± 33
HDL cholesterol (mg/dl)	58 ± 13	51 ± 14	50 ± 12*
HbA1c (mg/dl)	5.4 ± 0.8	6.2 ± 1.7*	5.8 ± 1.3
hsCRP (mg/dl)	0.12 ± 0.09	0.23 ± 0.15*	0.22 ± 0.13*
Prior myocardial infarction (%)		15	13
Lesion variables			
LAD intervention (%)		67	51
ACC/AHA lesion type B2/C (%)		66	42
Infarct-related artery (%)		50	31†
Multivessel disease (%)		36	37
Multivessel intervention (%)		10	10
Multilesion intervention (%)		31	11‡
Chronic total occlusion (%)		5	5
Number of stents		1.3 ± 0.4	1.1 ± 0.3
Coil stent (%)		9	7
Measurement with quantitative coronary angiography			
Stented segment length (mm)		19 ± 4.5	17 ± 4.7†
Reference diameter (mm)		2.97 ± 0.48	3.05 ± 0.73
MLD before PCI (mm)		0.59 ± 0.05	0.64 ± 0.59
MLD after PCI (mm)		2.35 ± 0.48	2.52 ± 0.67
Lesion length (mm)		12.7 ± 4.37	10.9 ± 4.03†

*$p < 0.05$ versus control subjects, †$p < 0.05$, ‡$p < 0.01$ versus with ISR; hypertension, ≥140/90 mm Hg or taking an antihypertensive medication; smoking, ≥10 cigarettes/day for ≥10 years; diabetes mellitus, defined according to the American Diabetes Association report or as taking an antidiabetic medication. Data are expressed as mean ± SD or percentage of the patients.

ACC/AHA = American College of Cardiology/American Heart Association; HDL = high-density lipoprotein; HbA1c = glycosylated hemoglobin; hsCRP = high-sensitivity C-reactive protein; ISR = in-stent restenosis; LAD = left anterior descending; LDL = low-density lipoprotein; MLD = minimal lumen diameter; PCI = percutaneous coronary intervention.

among three groups. Comparisons of FMD in patients with coronary stenting were performed with two-way analysis of variance for repeated measures, followed by post-hoc testing with a Scheffe test. The correlation of FMD with risk factor profiles was examined by linear regression analysis. The assessment of independent association of late ISR and TLR with the impairment of the follow-up FMD was performed with multivariate logistic regression analysis that included the following factors as categorical variables: impairment of the follow-up FMD (≤4.8%, obtained from receiver-operator characteristic analysis of FMD in the study patients), chest pain, positive exercise ECG test, infarct-related artery, lesion length (≥10 mm, arbitrarily defined as the 50th percentile of the distribution of the length in the study patients), multiple lesions with stenting, stented segment length (≥18 mm, arbitrarily defined as the 50th percentile of the distribution of the length in the study patients). All these variables had a significant relationship with late ISR in the Student unpaired *t* test or chi-square analysis. Statistical significance was defined as $p < 0.05$. Analyses were assessed, in part, with StatView 5.0 for Windows (Tokyo, Japan).

RESULTS

Comparisons of clinical characteristics between patients with and without ISR. In-stent restenosis was found in 46 (33%) patients at the follow-up coronary angiography. Patients with and without ISR had comparable clinical characteristics, including coronary risk factors and frequencies of each of the cardiac medications before PCI and during the six-month follow-up (Tables 1, 2, and 3). Also, frequencies of newly started or increased cardiac medications immediately after PCI were not significantly different between patients with and without ISR (Table 2). Levels of total cholesterol, high-density lipoprotein cholesterol (HDL-C), glycosylated hemoglobin (HbA1c), high-sensitivity C-reactive protein (hsCRP), systolic blood pressure, and body mass index at follow-up were improved compared with baseline in both groups of patients, and there was a reduction in the number of smokers and an increase in leisure-time physical activity, mainly by walking (≥30 min/day increase from baseline activity for >1 month) during the treatment periods in both groups (Table 3). These favorable changes, however, were not significantly different between patients with and without ISR (Table 3). During the six-month follow-up period, chest pain occurred in 21 (46%) of 46 patients with ISR and in 12 (13%) of the 95 patients without ISR ($p < 0.01$), as shown in Table 3. Although all of the study patients had the follow-up angiography and the discontinuation of the vasodilators before the measurements, according to the study protocol, there was neither an adverse complication nor refractory myocardial ischemia associated with the examinations in any patients.

Table 2. Comparisons of Frequency in Use of Medications Before PCI, Newly Added or Increased Immediately After PCI, and Medications During the Follow-Up Period

	With ISR (n = 46)	Without ISR (n = 95)
Medications before PCI		
Statin (%)	14	10
ACE-I (%)	6	10
ARB (%)	11	9
Calcium channel blocker (%)	20	22
Beta-blocker (%)	4	5
Aspirin (%)	50	42
Ticlopidine (%)	39	34
Sulfonylurea (%)	11	7
Insulin (%)	4	2
Medications added or increased after PCI		
Statin (%)	26	23
ACE-I (%)	26	33
ARB (%)	17	14
Calcium channel blocker (%)	43	45
Beta-blocker (%)	22	23
Aspirin (%)	50	58
Ticlopidine (%)	51	58
Sulfonylurea (%)	2	3
Insulin (%)	4	2
Medications during the follow-up period		
Statin (%)	38	32
ACE-I (%)	31	42
ARB (%)	28	19
Calcium channel blocker (%)	62	65
Beta-blocker (%)	26	27
Aspirin (%)	100	100
Ticlopidine (%)	90	92
Sulfonylurea (%)	13	10
Insulin (%)	9	4

There was no statistical difference in frequencies of medications between the two groups.

ACE-I = angiotensin-converting enzyme inhibitors; ARB = angiotensin receptor blockers; ISR = in-stent restenosis; PCI = percutaneous coronary intervention.

Quantitative coronary angiography. Patients with ISR had a higher prevalence of infarct-related artery and multiple lesions with stenting, longer stented segment length, and longer lesion length at the time of the stenting than those without ISR (Table 1). Target lesions revascularization at the six-month follow-up angiography was performed with PCI in 36 (78%) patients with ISR. Percutaneous coronary intervention for coronary segments other than the stented segments was performed at the six-month follow-up angiography in 5 (11%) patients with ISR and 8 (8%) patients without ISR (p = NS).

Exercise treadmill ECG test. The exercise ECG test was performed in 41 (89%) patients with ISR and 75 (79%) patients without ISR (p = NS). Among patients who had the exercise stress test, the test was positive in 22 (54%) patients with ISR and 17 (23%) patients without ISR ($p < 0.01$). Chest pain during the exercise test occurred in 15 (37%) patients with ISR and 5 (7%) patients without ISR ($p < 0.01$).

FMD. The initial FMD within three days before the coronary stenting was comparable between patients with