

# Plasma Concentrations of a Novel, Adipose-Specific Protein, Adiponectin, in Type 2 Diabetic Patients

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**Abstract**—Adiponectin is a novel, adipose-specific protein abundantly present in the circulation, and it has antiatherogenic properties. We analyzed the plasma adiponectin concentrations in age- and body mass index (BMI)-matched nondiabetic and type 2 diabetic subjects with and without coronary artery disease (CAD). Plasma levels of adiponectin in the diabetic subjects without CAD were lower than those in nondiabetic subjects ( $6.6 \pm 0.4$  versus  $7.9 \pm 0.5$   $\mu\text{g/mL}$  in men,  $7.6 \pm 0.7$  versus  $11.7 \pm 1.0$   $\mu\text{g/mL}$  in women;  $P < 0.001$ ). The plasma adiponectin concentrations of diabetic patients with CAD were lower than those of diabetic patients without CAD ( $4.0 \pm 0.4$  versus  $6.6 \pm 0.4$   $\mu\text{g/mL}$ ,  $P < 0.001$  in men;  $6.3 \pm 0.8$  versus  $7.6 \pm 0.7$   $\mu\text{g/mL}$  in women). In contrast, plasma levels of leptin did not differ between diabetic patients with and without CAD. The presence of microangiopathy did not affect the plasma adiponectin levels in diabetic patients. Significant, univariate, inverse correlations were observed between adiponectin levels and fasting plasma insulin ( $r = -0.18$ ,  $P < 0.01$ ) and glucose ( $r = -0.26$ ,  $P < 0.001$ ) levels. In multivariate analysis, plasma insulin did not independently affect the plasma adiponectin levels. BMI, serum triglyceride concentration, and the presence of diabetes or CAD remained significantly related to plasma adiponectin concentrations. Weight reduction significantly elevated plasma adiponectin levels in the diabetic subjects as well as the nondiabetic subjects. These results suggest that the decreased plasma adiponectin concentrations in diabetes may be an indicator of macroangiopathy. (*Arterioscler Thromb Vasc Biol.* 2000;20:1595-1599.)

**Key Words:** adiponectin ■ diabetes mellitus ■ coronary artery disease ■ adipose tissue

Atherosclerotic cardiovascular complications are the major causes of morbidity and mortality in type 2 diabetic patients.<sup>1</sup> The precise mechanism underlying the development of atherosclerotic vascular disease has not been fully elucidated. Abnormalities in lipid metabolism and hemostatic factors and the presence of insulin resistance are thought to contribute to atherosclerotic vascular damage in diabetes. Plasma plasminogen activator inhibitor type 1 (PAI-1) concentrations are elevated in type 2 diabetic patients, and increased plasma PAI-1 reduces fibrinolytic activity, which may play a role in the development of vascular complications.<sup>2,3</sup> Recent experimental and clinical studies have revealed that adipose tissue, especially intra-abdominal visceral adipose tissue, is an important source of plasma PAI-1.<sup>4,5</sup> Traditionally, adipose tissue has been considered to be an organ that passively stores excess energy as fat. Substantial research has explored the notion that adipose tissue secretes a variety of biologically active molecules, including cytokines,

growth factors, and complement factors, into the circulation.<sup>6-9</sup> Tumor necrosis factor (TNF)- $\alpha$  decreases tyrosine kinase activity of the insulin receptor and is overproduced in adipose tissues in insulin-resistant rodents and humans, suggesting that it is a possible mediator of insulin resistance in obesity and diabetes.<sup>6,10,11</sup> Leptin is produced specifically by adipose tissue and transmits a satiety signal to the central nervous system.<sup>7</sup> The molecule also affects glucose metabolism and insulin sensitivity.<sup>12,13</sup> These data suggest that the multiple molecules produced by adipose tissue contribute to the development of insulin resistance and atherosclerotic complications in diabetes mellitus.

Adiponectin is a novel, adipose-specific protein belonging to the collectin family.<sup>14,15</sup> The protein is present abundantly in the circulation, accounting for  $\approx 0.01\%$  of total plasma protein.<sup>16</sup> When the endothelium of the carotid arteries is injured by a balloon catheter in rats, adiponectin accumulates in the vascular walls.<sup>17</sup> Recently, we observed that adiponec-

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TABLE 1. Characteristics of the Subjects

	Men			P (ANOVA)	Women			P (ANOVA)
	Nondiabetic	Diabetic Without CAD	Diabetic With CAD		Nondiabetic	Diabetic Without CAD	Diabetic With CAD	
n	54	82	45		28	37	19	
Age, y	56±1	57±1	58±1	NS	58±2	61±1	62±2	NS
BMI, kg/m <sup>2</sup>	23.3±0.4	24.1±0.3	23.6±0.4	NS	24.0±1.0	23.6±0.5	24.6±0.9	NS
Fasting glucose, mmol/L	5.2±0.1	8.5±0.2	8.7±0.5	<0.001	5.1±0.1	8.5±0.4	8.6±0.5	<0.001
Fasting insulin, pmol/L	32±2	60±5	70±19	<0.05	35±5	60±8	78±19	<0.05
Total cholesterol, mmol/L	5.01±0.12	5.34±0.10	5.19±0.17	NS	5.65±0.33	5.31±0.13	5.65±0.30	NS
Triglycerides, mg/dL	156±21	176±11	193±24	NS	116±12	142±10	112±11	NS
HDL cholesterol, mmol/L	1.33±0.06	1.29±0.04	0.98±0.07	<0.001	1.46±0.07	1.43±0.09	1.37±0.09	NS
Fasting adiponectin, μg/mL	7.9±0.5	6.6±0.4	4.0±0.4	<0.001	11.7±1.0	7.6±0.7	6.3±0.8	<0.001
Fasting leptin, ng/mL	2.8±0.3	3.7±0.3	3.5±0.3	NS	11.1±2.0	9.4±0.9	13.3±2.5	NS

NS, not significant. Data are mean±SEM.

tin suppressed the attachment of monocytes to endothelial cells,<sup>18</sup> which is an early event in atherosclerotic vascular change. Adiponectin may have a role in protection against vascular damage. Although the expression of adiponectin mRNA is restricted in adipose tissue, its plasma concentrations are decreased in obesity.<sup>16</sup> The significance of adiponectin in diabetes mellitus has not been investigated. Plasma adiponectin may be dysregulated in disorders susceptible to atherosclerotic vascular diseases, such as diabetes mellitus. In this report, we investigated the plasma adiponectin concentrations in type 2 diabetic humans, especially with respect to atherosclerotic coronary vascular complications.

## Methods

### ELISA of Plasma Adiponectin and Leptin

All blood samples were drawn after an overnight fast unless otherwise indicated. Plasma samples were kept at  $-80^{\circ}\text{C}$  for subsequent assay. The concentration of plasma adiponectin was determined by ELISA as described previously.<sup>16</sup> The plasma leptin levels were also determined by ELISA. Briefly, recombinant human leptin cloned into pET3a (Novagen Inc) was expressed in *Escherichia coli* BL21 (DE3) (Novagen Inc). Monoclonal and polyclonal anti-leptin antibodies were produced against the recombinant leptin. Ten microliters of plasma was applied to a 96-well microtiter plate coated with mouse anti-leptin monoclonal antibody. The wells were washed and further incubated with rabbit anti-leptin polyclonal antibody, followed by incubation with horseradish peroxidase-labeled anti-rabbit IgG. Each well was reacted with *o*-phenylenediamine dihydrochloride (Sigma), and the absorbance at 492 nm was measured. Recombinant human leptin protein as described above was used as the standard.

### Determination of Plasma Adiponectin Levels in Nondiabetic and Diabetic Subjects

Blood specimens were obtained before and 2 hours after ingestion of a 75-g glucose load for assay of plasma glucose concentrations. Diabetes was diagnosed according to World Health Organization (1985) criteria. Plasma glucose was measured by a glucose oxidase method. A total of 183 diabetic patients including 127 men and 56 women were studied. The diabetic patients who suffered from myocardial infarction or who showed ischemic electrocardiographic changes on exercise testing with or without cardiac symptoms underwent coronary angiography. Among them, 64 (45 men and 19 women) were angiographically diagnosed as having CAD. The criteria of CAD was the reduction in luminal diameter of a major coronary artery branch by  $>75\%$ . Forty-four patients (21 men and 23 women) had retinopathy, which was diagnosed by an ophthalmolo-

gist. Sixty patients (42 men and 18 women) had nephropathy, with a urinary albumin concentration of  $>20$  mg/g creatinine. Urinary albumin concentrations were determined by radioimmunoassay and adjusted to urinary creatinine concentrations. Eighty-two age- and body mass index (BMI)-matched subjects with normal glucose tolerance, including 54 men and 28 women, were selected from healthy volunteers who underwent a medical examination and served as controls (Table 1). Subjects with impaired glucose tolerance were excluded from the study. All women studied herein were postmenopausal. Informed consent was obtained from all subjects.

Serum cholesterol and triglyceride concentrations were determined by enzymatic methods. HDL cholesterol was also measured by an enzymatic method after heparin and calcium precipitation. The value of hemoglobin A1c was determined by high-performance liquid chromatography.

### Change in Plasma Adiponectin Concentrations Before and After Weight Reduction

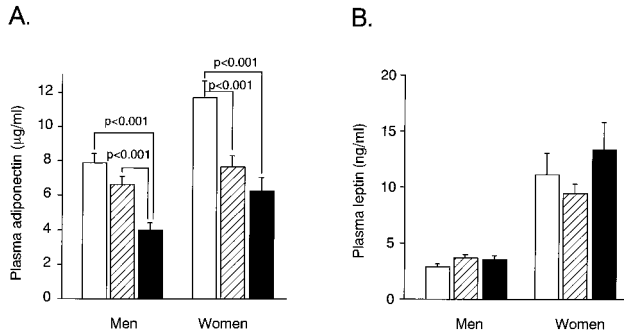
To study the effect of weight reduction on plasma adiponectin levels, 13 nondiabetic, obese subjects (6 men and 7 women; BMI,  $36.8\pm 1.2$  kg/m<sup>2</sup>; age,  $45\pm 5$  years) and 9 diabetic, obese subjects (6 men and 3 women; BMI,  $34.8\pm 2.6$  kg/m<sup>2</sup>; age,  $50\pm 3$  years) were hospitalized and placed on a calorie-restricted diet to reduce their body weight. The weight reduction therapy was performed according to the calorie restriction program in our clinic. In brief, starting at 2000 kcal/d, total calorie intake was decreased sequentially ( $-400$  kcal/d for 2 weeks) to 800 kcal/d (carbohydrate 50%, fat 25%, and protein 25%), and this calorie level was maintained. Their BMI decreased significantly within 2 months (nondiabetic,  $33.2\pm 1.0$  kg/m<sup>2</sup>,  $P<0.001$ ; diabetic,  $30.4\pm 2.0$  kg/m<sup>2</sup>,  $P<0.01$ ). Mean BMI changes of  $10\pm 1\%$  in nondiabetic subjects and of  $12\pm 2\%$  in diabetic subjects were achieved. The plasma was obtained before and at the end of the weight reduction period.

### Daily Profile of Plasma Adiponectin Concentrations

The circadian variation in plasma adiponectin concentrations was investigated in 7 nondiabetic subjects (3 men and 4 women) and 6 diabetic subjects (4 men and 2 women). Their BMI was  $31.1\pm 2.4$  kg/m<sup>2</sup> (range, 24.5 to 39.3 kg/m<sup>2</sup>) in nondiabetic subjects (age,  $54\pm 5$  years) and  $33.0\pm 3.5$  kg/m<sup>2</sup> (range, 22.4 to 44.8 kg/m<sup>2</sup>) in diabetic subjects (age,  $54\pm 8$  years). Each subject was hospitalized and received breakfast at 7 AM, lunch at noon, and dinner at 6 PM. Plasma was obtained from each subject at 6:30, 9:30, and 11:30 AM and at 2:30, 5:30, 8:30, and 9:30 PM. The plasma levels of glucose, insulin, adiponectin, and leptin were determined.

### Statistics

Data are expressed as mean±SEM. Intergroup differences in the parameters were analyzed by *t* test. Significant group differences and daily changes in the plasma adiponectin and leptin levels were



**Figure 1.** Plasma levels of adiponectin and leptin in normal and diabetic patients with and without CAD. The plasma levels of adiponectin (A) and leptin (B) in normal and diabetic patients without and with CAD were determined as described in Methods. Data are presented as mean  $\pm$  SEM.

compared by one-way ANOVA and tested further by the Fisher multiple comparison method. Linear relationships between key variables were tested by Pearson's correlation coefficient. Multiple linear regression analysis was performed to evaluate the independent relationship of the studied variables.

## Results

### Plasma Adiponectin Levels in Nondiabetic and Diabetic Subjects With and Without CAD

Plasma levels of the adipose-specific proteins adiponectin and leptin were measured in 183 patients with type 2 diabetes and in 82 age- and BMI-matched healthy subjects with normal glucose tolerance. There was no significant difference in the plasma levels of leptin between diabetic and nondiabetic groups. In contrast, plasma levels of adiponectin were significantly lower in the diabetic than in the nondiabetic subjects in both men and women ( $5.7 \pm 0.3$  versus  $7.9 \pm 0.5$   $\mu\text{g/mL}$  in men,  $P < 0.001$ ;  $7.2 \pm 0.5$  versus  $11.7 \pm 1.0$   $\mu\text{g/mL}$  in women,  $P < 0.001$ ). The diabetic subjects were subdivided into 2 groups, those with and without CAD. Plasma adiponectin concentrations in diabetic women without CAD were significantly lower than those in nondiabetic women ( $7.6 \pm 0.7$  versus  $11.7 \pm 1.0$   $\mu\text{g/mL}$ ,  $P < 0.001$ ). Diabetic women with CAD exhibited even lower plasma adiponectin concentrations (Table 1 and Figure 1). In men, diabetic subjects without CAD also showed lower plasma adiponectin levels compared with nondiabetic subjects ( $6.6 \pm 0.4$  versus  $7.9 \pm 0.5$   $\mu\text{g/mL}$ ,  $P = 0.07$ ), although this difference was not statistically significant. Plasma adiponectin levels in diabetic men with CAD were even lower and statistically significant

when compared with diabetic men without CAD ( $4.0 \pm 0.4$  versus  $6.6 \pm 0.4$   $\mu\text{g/mL}$ ,  $P < 0.001$ ). No significant difference was found in plasma leptin concentrations between diabetic subjects with and without CAD. The plasma adiponectin levels did not differ between patients with and without retinopathy ( $6.1 \pm 0.9$  versus  $5.5 \pm 0.5$   $\mu\text{g/mL}$  in men,  $7.6 \pm 0.9$   $\mu\text{g/mL}$  versus  $6.9 \pm 0.8$   $\mu\text{g/mL}$  in women) nor in those with and without nephropathy ( $5.2 \pm 0.6$  versus  $5.8 \pm 0.7$   $\mu\text{g/mL}$  in men,  $6.1 \pm 0.9$   $\mu\text{g/mL}$  versus  $7.4 \pm 0.9$   $\mu\text{g/mL}$  in women). There was no correlation between plasma adiponectin levels and urinary albumin concentrations (men,  $r = 0.11$ ; women,  $r = 0.14$ ).

Next we analyzed the correlation between plasma levels of adiponectin and other parameters. Plasma levels of adiponectin were negatively correlated with BMI ( $r = -0.29$ ,  $P < 0.001$ ) as previously reported.<sup>16</sup> No significant correlation was found between the plasma levels of adiponectin and age ( $r = 0.05$ ). The plasma concentration of adiponectin was negatively correlated with plasma glucose level ( $r = -0.26$ ,  $P < 0.001$ ), the value of hemoglobin A1c ( $r = -0.23$ ,  $P < 0.001$ ), and plasma insulin level ( $r = -0.18$ ,  $P < 0.01$ ). Among the lipid parameters, the serum triglyceride level was negatively correlated ( $r = -0.32$ ,  $P < 0.001$ ) and the HDL cholesterol level positively correlated ( $r = 0.35$ ,  $P < 0.001$ ) with the plasma adiponectin level. Total cholesterol level was not correlated with plasma adiponectin level ( $r = 0.12$ ). A multiple linear regression analysis revealed that the presence of diabetes and CAD was significantly associated with the decreased plasma level of adiponectin, independent of BMI in women. In men, the presence of CAD but not of diabetes was significantly associated with the decreased plasma adiponectin concentration. Plasma insulin and HDL cholesterol levels were not independent parameters associated with the decreased plasma adiponectin concentration. Serum triglyceride concentration remained an independent parameter to the plasma adiponectin level (Table 2).

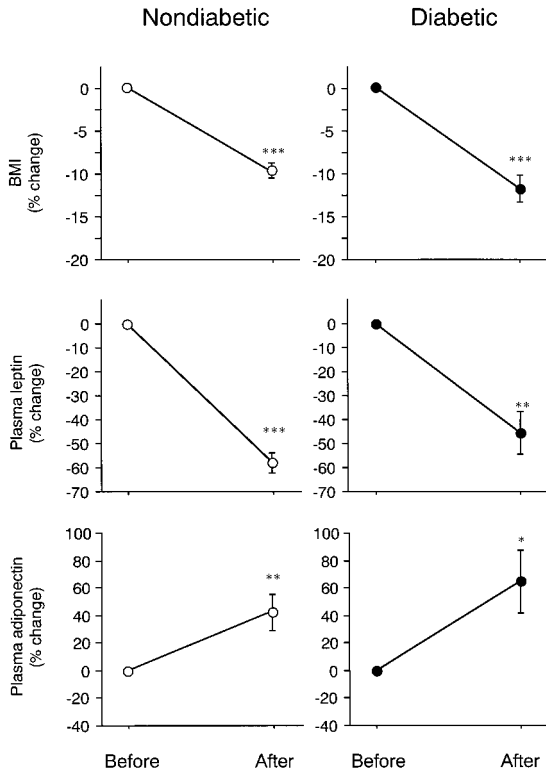
### Regulation of Plasma Adiponectin Levels in Diabetic Patients

Fasting plasma levels of adiponectin were decreased in diabetic subjects. Regulation of plasma levels of adiponectin may be disturbed in the diabetic state. It is well known that plasma leptin levels are regulated by adiposity, and they are reduced by weight reduction.<sup>19,20</sup> We investigated the effect of weight reduction on plasma adiponectin concentrations in both nondiabetic and diabetic subjects. An  $\approx 10\%$  reduction

**TABLE 2. Multiple Linear Regression Analysis for Plasma Adiponectin**

Independent Variable	Men			Women		
	Regression Coefficient	SE of Regression Coefficient	P	Regression Coefficient	SE of Regression Coefficient	P
Intercept	13.8	2.74	<0.001	19.9	3.86	<0.001
BMI, kg/m <sup>2</sup>	-0.297	0.104	<0.01	-0.298	0.117	<0.05
Plasma insulin, pmol/L	0.0003	0.025	NS	-0.097	0.066	NS
Triglycerides, mg/dL	-0.006	0.002	<0.05	-0.020	0.009	<0.05
HDL cholesterol, mmol/L	0.037	0.020	NS	0.030	0.030	NS
Diabetes, yes/no	-0.866	0.620	NS	-3.007	1.065	<0.01
CAD, yes/no	-2.347	0.758	<0.01	-2.810	1.332	<0.05

NS, not significant.  $R^2 = 28\%$  in men,  $46\%$  in women.



**Figure 2.** Changes in plasma levels of leptin and adiponectin during weight reduction. Plasma levels of adiponectin and leptin were determined in 13 nondiabetic subjects (open circles) and 9 diabetic subjects (closed circles) as described in Methods. Plasma levels of leptin and adiponectin were expressed as percent change from initial values. Values shown are mean  $\pm$  SEM. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for comparison with initial values.

of BMI (nondiabetics,  $-10 \pm 1\%$ ; diabetics,  $-12 \pm 2\%$ ) was achieved in 13 nondiabetic and 9 diabetic subjects. Plasma leptin levels were decreased in both nondiabetic ( $-58 \pm 4\%$ ,  $P < 0.001$ ) and diabetic ( $-46 \pm 9\%$ ,  $P < 0.01$ ) subjects. On the other hand, plasma adiponectin significantly increased after body weight reduction in nondiabetic subjects ( $42 \pm 13\%$ ,  $P < 0.01$ ). An equivalent increase was observed in diabetic subjects ( $65 \pm 22\%$ ,  $P < 0.05$ ; Figure 2). Therefore, the plasma adiponectin level is negatively regulated by adiposity. Regulation by adiposity was conserved in the diabetic subjects.

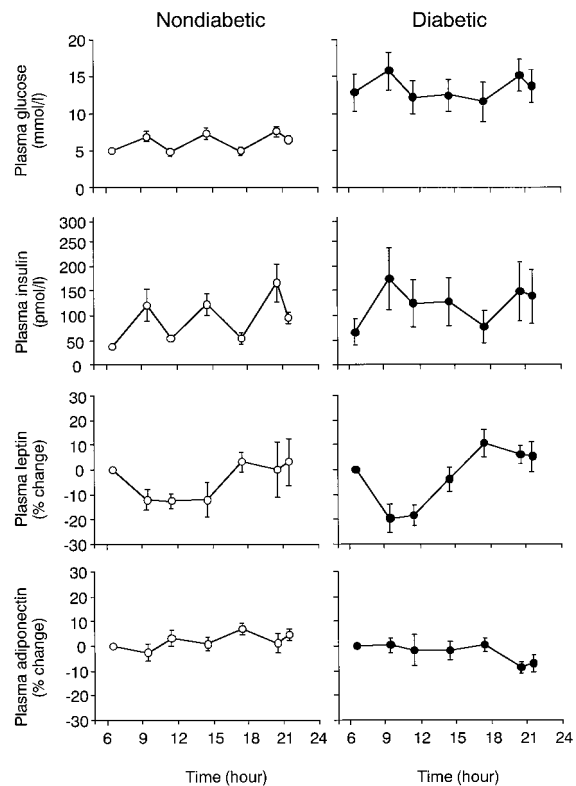
Plasma leptin levels are known to show circadian variation: they are low in the morning and high in the evening.<sup>21</sup> The daily profile of plasma adiponectin was investigated. Plasma glucose and insulin levels were elevated after every meal. Plasma leptin level showed a single peak at 6 PM. Plasma adiponectin showed no daily change in both the nondiabetic and diabetic subjects (Figure 3).

### Discussion

Adiponectin is a collagen-like protein produced specifically by adipose tissue and is abundantly present in the circulation. When the vascular endothelium is injured, adiponectin accumulates in the subintimal space of the arterial wall through its interaction with collagens in the vascular intima.<sup>17</sup> Adiponectin attenuates TNF- $\alpha$ -induced expression of adhesion molecules in endothelial cells,<sup>18</sup> which is an initial step of atherosclerosis.<sup>22,23</sup> The significance of adiponectin in human disease has not been fully elucidated. In a previous study, we

showed that obese subjects and subjects with CAD exhibited decreased plasma concentrations of adiponectin.<sup>16,18</sup> In the present study, we investigated the plasma adiponectin concentrations in subjects with type 2 diabetes mellitus.

The subjects with type 2 diabetes mellitus showed significantly decreased plasma adiponectin concentrations. Although plasma adiponectin levels are negatively correlated with BMI, diabetic subjects had lower values of plasma adiponectin than did nondiabetic subjects, independent of BMI. Insulin regulates the secretion of various proteins from adipose tissue. Elevated plasma insulin in the diabetic subjects in this study may have been responsible for the decreased plasma adiponectin concentrations. The plasma level of leptin, another molecule specifically secreted from adipocytes, was positively correlated with fasting plasma insulin.<sup>7,19</sup> On the other hand, the plasma adiponectin concentration was negatively correlated with the fasting plasma insulin level. The daily profile of plasma adiponectin levels revealed that it was not affected by food intake, in contrast with increased plasma insulin levels, suggesting that insulin does not have an acute effect on the plasma adiponectin level. Chronic insulin resistance in type 2 diabetes may be related to decreased plasma adiponectin. Overproduction of TNF- $\alpha$  by adipose tissue has been suggested in the development of insulin resistance.<sup>6,10,11</sup> Adiponectin interferes with TNF- $\alpha$  signaling in endothelial cells.<sup>18</sup> Decreased plasma adiponectin may play a causative role in the development of insulin



**Figure 3.** Daily profiles of plasma glucose, insulin, leptin, and adiponectin concentrations. Plasma levels of glucose, insulin, leptin, and adiponectin were determined in 7 nondiabetic (open circles) and 6 diabetic (closed circles) subjects as described in Methods. Plasma levels of leptin and adiponectin were expressed as percent change from fasting values (ie, at 6:30 AM). Each point represents mean  $\pm$  SEM.



resistance. The effect of adiponectin on TNF- $\alpha$  signaling and insulin sensitivity in muscles should be investigated.

Another remarkable finding in this study was that plasma adiponectin levels were decreased prominently in diabetic subjects with CAD. In contrast, plasma levels of leptin did not differ between the diabetic subjects with and without CAD. Experimental research has indicated that adiponectin has potential antiatherogenic properties.<sup>17,18</sup> Thus, the decreased plasma adiponectin in diabetic subjects may play a role in the development of atherosclerotic vascular damage. Another possibility is that accumulation of adiponectin in atherosclerotic vascular walls may accelerate its half-life in plasma, resulting in the reduction of the plasma concentration of adiponectin in subjects with CAD. The causal relationship between atherosclerotic vascular disease and decreased plasma levels of adiponectin cannot be derived from our cross-sectional study. Further experimental cell research and prospective clinical studies will be necessary to clarify these points.

In the current study, the plasma adiponectin level was independently correlated with the serum triglyceride level by multiple regression analysis. Hypertriglyceridemia is 1 of the major clinical features of the insulin resistance syndrome and is often accompanied by elevated plasma PAI-1 levels. Hypertriglyceridemia may take part in the development of atherosclerosis in concert with the dysregulation of adipocyte-derived proteins, such as elevated PAI-1 and hypoadiponectinemia.

The reason for the sex difference in adiponectin concentration in the diabetic subjects without CAD has not been made clear. Clinically normal women have higher adiponectin levels than do men, as previously reported.<sup>16</sup> Sex hormones, including estrogen, progesterone, and androgen, may affect the plasma adiponectin level. However, all of the women in this study were postmenopausal. Thus, the sexual dimorphism in adiponectin level cannot be accounted for solely by the effect of estrogen and/or progesterone. Diabetic patients with CAD are often asymptomatic. The diabetic women in this study may include patients with latent atherosclerotic vascular diseases. Another possibility is that women with hypoadiponectinemia are susceptible to the development of insulin resistance and type 2 diabetes. It is necessary to investigate the effect of adiponectin on insulin signaling and glucose metabolism.

Different from leptin, the new adipocyte-derived protein adiponectin could be an indicator of macroangiopathy associated with diabetes. A lower adiponectin level may increase the risk of atherosclerosis. Reduction of BMI resulted in elevation of the plasma adiponectin. Consequently, attempts to reduce body weight to normalize the plasma adiponectin levels could be effective in preventing the development of atherosclerosis. However, this requires confirmation by means of prospective studies.

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