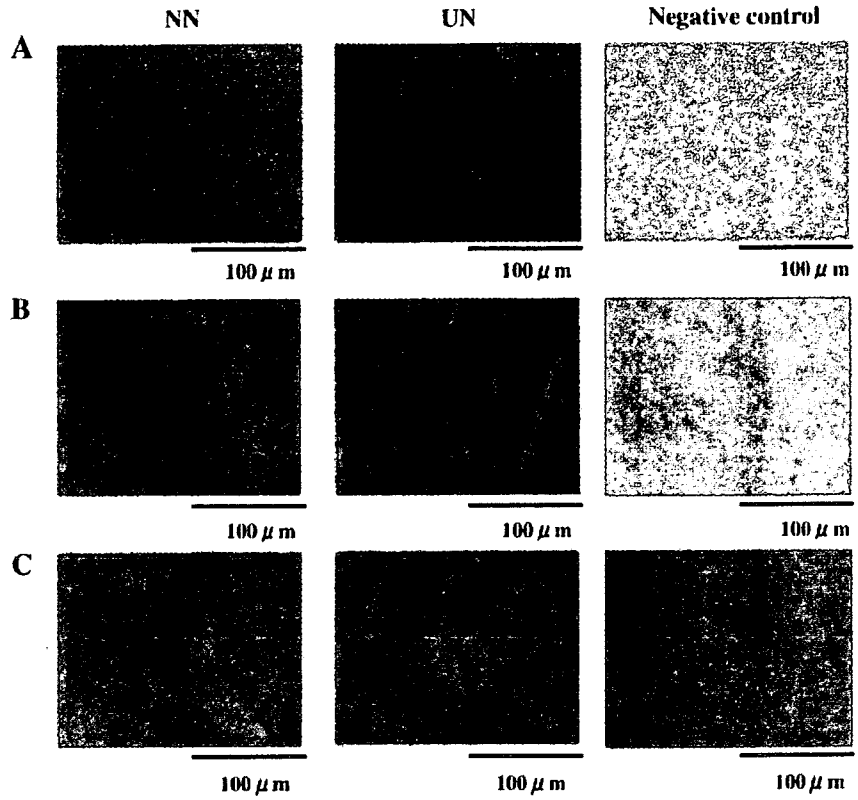

FIG. 5. Immunohistochemistry for angiotensin II (A), ET-1 (B), and renin (C) in the left ventricles of NN offspring (*left panels*) and UN offspring (*middle panels*) at 16 wk. Negative controls of NN offspring using normal rabbit serum (for angiotensin II and ET-1) or goat serum for renin are shown in *right panels*. Original magnification was ×400.

Using the same animal model, we recently reported pronounced obesity in UN offspring on a high-fat diet compared with NN offspring (9). We found premature onset of the neonatal leptin surge, *i.e.* a transient increase in serum leptin levels during the neonatal period, in UN offspring. We also demonstrated that the premature leptin surge programs hypothalamic low sensitivity to circulating leptin, a potent antiobesity hormone, causatively contributing to pronounced obesity on a high-fat diet in adulthood, by showing that an artificial premature leptin surge model produced hypothalamic low sensitivity to circulating leptin and pronounced obesity on a high-fat diet (9). However, in the present study, an artificial premature leptin surge did not increase SBP in NN offspring. Moreover, artificial premature leptin surge did not augment cardiac remodeling (Kawamura, M., and H. Itoh, unpublished observations). We also revealed that chemical injury of the ARC by neonatal monosodium glutamate treatment during the neonatal period cancelled the acceleration of obesity on the high-fat diet in UN offspring (9).

TABLE 4. The mRNA expression of Ang, AT1R, AT2R, ACE, ET-1, ANP, and BNP in the murine fetal whole heart at 18.5 dpc

	NN (n = 10)	UN (n = 10)
Ang/GAPDH	0.38 ± 0.05	0.74 ± 0.17[a]
AT1R/GAPDH	0.25 ± 0.02	0.31 ± 0.03
AT2R/GAPDH	4.88 ± 0.55	6.53 ± 1.11
ACE/GAPDH	0.10 ± 0.01	0.18 ± 0.03[b]
ET-1/GAPDH	2.13 ± 0.22	3.22 ± 0.44[a]
ANP/GAPDH	17.32 ± 1.93	22.58 ± 2.63
BNP/GAPDH	0.811 ± 0.07	0.70 ± 0.04

Values are the mean ± SEM (arbitrary units).
[a] $P < 0.05$; [b] $P < 0.01$ *vs.* NN offspring.

However, a significant increase in SBP was not blocked by monosodium glutamate treatment in the present study. The mechanisms leading to increased blood pressure in adult UN offspring with undernutrition *in utero* are currently not entirely clear.

There were no significant changes in the mRNA expression of cardiac RAS-associated bioactive substances at 3 wk (Figs. 3 and 4). On the other hand, at 8 wk, the mRNA expression of ET-1, a factor promoting cardiac remodeling (18, 19), was significantly elevated in the left ventricles of UN offspring (Fig. 4A). However, several anticardiac remodeling phenomena were observed at the same time in the left ventricles as follows. The Ang mRNA expression was significantly decreased (Fig. 3A), concomitantly with the significant increase of AT2R (Fig. 3D), which suppresses cardiac remodeling (20). ANP and BNP are secreted from the heart and antagonize RAS through a decrease in blood pressure, diuresis, anticardiac hypertrophy, and anticardiac fibrosis, *etc.* (21, 22). The significant elevation of BNP mRNA expression in the left ventricles of UN offspring at 8 wk, in parallel with a tendency for an increase in ANP mRNA expression, suggested protective effects on cardiac tissues against the acceleration of cardiac remodeling. Therefore, changes that both promote and suppress cardiac remodeling are simultaneously observed in the left ventricles of UN offspring at 8 wk. These findings lead us to speculate that a kind of compensatory mechanism might be operating, thereby protecting the heart from ominous cardiac transformation at 8 wk, which was relevant to the finding that neither cardiac hypertrophy (Table 3B) nor augmentation of perivascular

fibrosis (Fig. 2) was observed with a significant increase in SBP (Fig. 1).

At 16 wk, a significant augmentation of cardiac remodeling, *i.e.* cardiac hypertrophy (Table 3B) and perivascular fibrosis (Fig. 2), was observed in UN offspring. It is a further aim of the study to assess the movement and/or thickness of the ventricular wall by ultrasound examination.

In the present study, we first demonstrated that undernutrition *in utero* significantly increased the mRNA expression of both Ang (Fig. 3A) and ET-1 (Fig. 4A) in the left ventricles of UN offspring at 16 wk, concomitantly with the augmentation of cardiac hypertrophy and perivascular fibrosis. Angiotensin II is derived from Ang and plays a central role in the local cardiac RAS in the augmentation of cardiac remodeling (4, 5). ET-1 has been found to induce hypertrophy of cardiomyocytes (18), as well as cardiac fibrosis (19). ET-1 has a close association with the local cardiac RAS in the process of cardiac remodeling (23, 24). In the present study, the significant elevation of both Ang and ET-1 mRNA levels in the left ventricle of UN offspring was observed at 16 wk. The immunostaining of both angiotensin II and ET-1 showed a tendency to increase in UN offspring compared with NN offspring at 16 wk. These findings suggested a possible decompensation of cardiac homeostasis in response to various portentous factors, as a result of fetal undernutrition, including an increase in blood pressure. A significant elevation in the AT2R mRNA expression, which suppresses cardiac remodeling by antagonizing the effects of signaling through the AT1R (20), was observed in UN offspring at 8 and 16 wk, but the increase relative to NN offspring was much lower at 16 wk than at 8 wk (Fig. 3D). Long-term observations are necessary to prove that 16 wk is the beginning of decompensation of cardiac homeostasis in this animal model. Nevertheless, these findings suggested a possible involvement of local cardiac RAS activation in the developmental origins of cardiac remodeling.

Rather stable expression was observed in ACE and AT1R after birth in UN offspring. Ang mRNA expression decreased at 8 wk and increased at 16 wk. More detailed molecular investigation is necessary to clarify the regulatory mechanism of each substance.

A few renin positive cells were detected in the left ventricle at 16 wk (Fig. 5C), although mRNA expression was below detection sensitivity of quantitative RT-PCR. This discrepancy was relevant to the recent observation that cardiac renin was predominantly derived from circulation (25). There was no apparent difference in cardiac renin immunostaining between NN and UN offspring at 16 wk. It is an interesting study to investigate whether cardiac renin uptake is involved in developmental origins of cardiac remodeling.

A significant augmentation of mRNA expression of Ang, ACE, and ET-1 was observed in the whole fetal heart at 18.5 dpc (Table 4). A possible association of these changes with local cardiac RAS activation in adulthood is a future aim of the study.

In summary, using a mouse model of fetal undernutrition, we here demonstrated the possible involvement of the local cardiac RAS in the developmental origins of cardiac disorders, represented by cardiac remodeling, by a longitudinal assessment of the expression of local cardiac RAS-associated bioactive substances from the fetal to adult periods. This study also highlighted the local cardiac RAS as a promising target for prophylactic intervention in the developmental origins of cardiovascular disease.

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Association between insulin resistance and endothelial dysfunction in type 2 diabetes and the effects of pioglitazone

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Abstract

Endothelial dysfunction is regarded as an early stage of atherosclerosis, and plays a role in the development of atherosclerotic diseases. Insulin resistance is related to the atherosclerotic process. In this study, we examined the association between endothelial function and insulin resistance in 48 subjects with type 2 diabetes. In addition, the effects of pioglitazone treatment on endothelial function and insulin resistance were investigated in a subgroup of subjects. Endothelial function of the brachial artery was non-invasively assessed using ultrasound technique. We measured flow-mediated endothelium-dependent vasodilation (FMD) and glyceryl trinitrate-induced endothelium-independent vasodilation (GTN). Insulin sensitivity was measured by the steady-state plasma glucose (SSPG) method. High SSPG levels indicate insulin resistance. There was a significant inverse correlation ($r = -0.462$, $p < 0.001$) between SSPG and FMD. Systolic blood pressure was inversely correlated with FMD ($r = -0.360$, $p < 0.013$). By multiple regression analysis, insulin resistance was the sole predictor of FMD. The effects of chronic treatment with pioglitazone were assessed in 10 subjects with type 2 diabetes. The increase in FMD significantly correlated with the decrease in SSPG. There is a significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone.

Keywords: Endothelial dysfunction; Insulin resistance; Pioglitazone

1. Introduction

Endothelial dysfunction is thought to be an important early feature in the development of atherosclerosis and occurs in subjects with type 2 diabetes mellitus [1–4]. Insulin resistance is also associated with atherosclerosis and is observed in subjects with type 2 diabetes [5,6]. We previously reported the association between endothelial dysfunction and insulin resistance in patients with essential hypertension [7]. However, the mechanisms responsible for endothelial dysfunction and insulin resistance in hypertension might be different from those of type 2 diabetes. Therefore, we evaluated the relationship between endothelial dysfunction and insulin resistance in patients with type 2 diabetes. Thiazolidinediones, an agonist for the peroxisome proliferator-activated receptor γ (PPARγ), improve insulin resistance. If there is a significant relationship

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between endothelial dysfunction and insulin resistance, thiazolidinediones might influence endothelial function. Therefore, we examined the effects of pioglitazone on endothelial dysfunction and insulin resistance in a subgroup of subjects with type 2 diabetes to verify the relationship between endothelial dysfunction and insulin resistance.

The main purpose of this study was to investigate the relation between vascular endothelial dysfunction and insulin resistance in type 2 diabetes. In addition, the influence of pioglitazone treatment was examined.

2. Subjects and methods

2.1. Subjects

Forty-eight (30 males and 18 females) patients with type 2 diabetes were recruited in the Department of Diabetes and Atherosclerosis of the National Cardiovascular Center. The subjects did not have diabetic retinopathy or nephropathy. Subjects were included on the basis of the following criteria: age between 40 and 79 years, body mass index (BMI) between 17 and 35 kg/m^2, type 2 diabetes confirmed by American Diabetes Association criteria [8]. Subjects were excluded from participation if they had coronary heart, peripheral vascular, renal, hepatic or other endocrine diseases. Subjects were excluded if they had a resting seated blood pressure greater than 150 mmHg systolic or greater than 90 mmHg diastolic, or were taking anti-hypertensive drugs. Diabetes duration was 5.3 ± 1.9 years (3–7 years). Diabetes treatment regimens included diet alone (27 subjects), sulfonylures (18 subjects) and metformin (3 subjects).

The 48 subjects had an average age of 64 ± 1 years, with a mean BMI of 24.6 ± 0.3 kg/m^2, HbA_{1c} of 8.6 ± 0.2%, total cholesterol of 199 ± 5 mg/dl, HDL-cholesterol of 43 ± 2 mg/dl and triglycerides of 137 ± 14 mg/dl. Mean systolic and diastolic blood pressures were 131 ± 3 and 74 ± 2 mmHg, respectively.

Of the 48 diabetic subjects, 10 subjects were started on a single 15 or 30 mg-tablet of pioglitazone (Actos, Takeda Pharmaceuticals, Tokyo, Japan) by mouth each day. Inclusion criteria of the pioglitazone treatment were male, non-smoker, diet alone treatment and mild to severe insulin resistance (SSPG > 160 mg/dl). They received a mean dose of 25.5 ± 2.3 mg/day (30 mg/day: seven subjects and 15 mg/day: three subjects) of pioglitazone for 16.3 ± 1.6 weeks (10–20 weeks). The secondary assessments of endothelial function and insulin sensitivity were performed after the pioglitazone treatments.

The study protocol was approved by the ethics committee of the National Cardiovascular Center. The experiments were conducted with the understanding and the consent of each participant.

2.2. Methods

2.2.1. Assessment of endothelial function

Using the ultrasound method, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasound measurements were carried out based on the method described by Celermajer et al. [9] and our method was reported previously [7]. The assessments were performed after an overnight fast in a quiet air-conditioned room (22–23 °C). The diameter of the brachial artery was measured on B-mode ultrasound images, with the use of a 10-MHz linear array transducer (ProSound SSD-5500, ALOKA, Tokyo, Japan). The right brachial artery was scanned in longitudinal sections 1–10 cm above the elbow, after at least 15 min of rest in the supine position. After the detection of the right transducer position, the skin surface was marked and the arm was kept in the same position during the study. All scans were recorded using a super-VHS videocassette recorder (SONY, SVO-9500MD), and analyzed later.

At first, baseline measurements of the diameter were carried out. Endothelium-dependent vasodilation (flow-mediated dilation) was determined by the scans during reactive hyperemia. Because flow-mediated vasodilation was mainly blocked by *N*-monomethyl-L-arginine (an inhibitor of endothelial nitric oxide synthase) this dilation was regarded as endothelium dependent [10]. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after deflation. After 10–15 min rest, the second control scan of the diameter and the flow velocity was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5–4 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to the posterior 'm' line (interface between the media and adventitia) at endo-diastole, coincident with the R wave on a continuously recorded electrocardiogram. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the flow-mediated dilation were carried out 45–60 s after the cuff release to measure a maximum diameter. Vasodilation by reactive hyperemia (flow-mediated dilation, FMD) or glyceryl trinitrate (GTN) was expressed as the percent change in diameter compared to the baseline values.

2.2.2. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated by a modified steady state plasma glucose (SSPG) method [6,7,11] using Sandostatin (octreotide acetate; Novartis, Basel, Switzerland) after an overnight fasting for at least 12 h. Sandostatin (9.8 pmol in bolus followed by a constant infusion of 73.5 pmol/h) and Novolin R insulin (Novo Nordisk S/A, Tokyo, Japan, 45 pmol/kg [7.5 mU/kg] in a bolus followed by a constant infusion at a rate of 4.62 pmol/kg/min [0.77 mU/kg/min]) were infused intravenously for 120 min.

Glucose in a final 12% solution containing KCl (0.5 μmol/kg/min) were infused at a rate of 0.033 mmol/kg/min [6 mg/kg/min] through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for determination of glucose and insulin. Value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels indicate peripheral insulin resistance. At 120 min SSPG was rapidly measured using a Glucometer (Bayer Corporation, Osaka, Japan) separate from the usual measurement of glucose and insulin. When rapidly measured, if SSPG was found to be lower than 250 mg/dl, oral glucose intake was necessary to prevent hypoglycemia after the insulin sensitivity test. The subjects should have lunch within 30 min after the insulin sensitivity test to prevent hypoglycemia. Homeostasis model assessment (HOMA-IR) was calculated from fasting glucose and insulin concentrations during insulin sensitivity test as follows: HOMA-IR = fasting glucose (mg/dl) × fasting insulin (μU/ml)/405.

2.3. Statistical analysis

Values are expressed as mean ± S.E. A probability value of <0.05 was considered to indicate statistical significance. The strength of the correlation between FMD and GTN with respect to risk factors was assessed by Pearson's linear correlation and multiple regression analysis. The effects of pioglitazone on each clinical parameter were assessed by paired *t*-test and Pearson's linear correlation.

3. Results

3.1. Association between endothelial dysfunction and each parameter in 48 subjects

A significant inverse correlation was observed between FMD and SSPG ($r = -0.462$, $p < 0.001$; Fig. 1). There was no relation between FMD and HbA_{1c} ($p = 0.856$). We also observed a significant inverse correlation between FMD and systolic blood pressure ($r = -0.360$, $p < 0.013$). No significant correlation was found between FMD and diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. There was no relationship between FMD and HOMA-IR ($p = 0.097$).

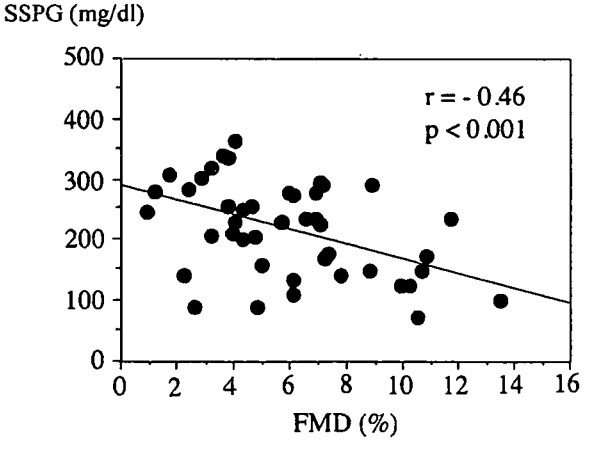

Fig. 1. Relationship between FMD and SSPG in subjects with type 2 diabetes. FMD, flow-mediated vasodilation; SSPG, steady state plasma glucose.

We performed multiple regression analysis to evaluate the independent influence of risk factors including SSPG, systolic blood pressure, HbA_{1c}, total cholesterol, BMI and age on FMD. FMD was independently related to SSPG (regression coefficient: $\beta = -0.419$, $p = 0.0086$) but not to systolic blood pressure ($\beta = -0.254$, $p = 0.0782$), HbA_{1c} ($\beta = -0.090$, $p = 0.5616$), total cholesterol ($\beta = -0.067$, $p = 0.6336$), BMI ($\beta = -0.258$, $p = 0.0863$) or age ($\beta = -0.085$, $p = 0.5650$).

With respect to GTN, no significant correlation was observed between GTN and SSPG or other parameters, including HbA_{1c}, diabetic duration, systolic blood pressure, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI.

3.2. Effects of pioglitazone treatment on endothelial function and insulin resistance

The effects of treatment with pioglitazone were assessed in 10 male subjects with type 2 diabetes (a subgroup of 48 subjects). Table 1 shows the clinical parameters of the 10 subjects before and after pioglitazone treatment. SSPG, HbA_{1c} and fasting plasma glucose decreased and FMD increased significantly due to pioglitazone treatment. However, BMI, total cholesterol, HDL-cholesterol, triglyceride, systolic blood pressure and diastolic blood pressure did not

Table 1
Clinical characteristics of the subjects with type 2 diabetes treated with pioglitazone

	Before Tx	After Tx
Number	10	
Age (years)	65 ± 2	
SSPG (mg/dl)	230 ± 13	185 ± 17*
FMD (%)	4.5 ± 1.1	8.1 ± 1.5***
Body mass index (kg/m^2)	24.4 ± 0.4	24.7 ± 0.4
Fasting plasma glucose (mg/dl)	162 ± 11	133 ± 8*
HbA_{1c} (%)	8.4 ± 0.4	7.0 ± 0.3**
Total cholesterol (mg/dl)	199 ± 8	206 ± 7
HDL cholesterol (mg/dl)	47 ± 4	50 ± 4
Triglyceride (mg/dl)	120 ± 15	129 ± 13
Systolic blood pressure (mmHg)	137 ± 5	137 ± 2
Diastolic blood pressure (mmHg)	78 ± 5	79 ± 1

Values are mean ± S.E. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ vs. before Tx. Tx, Treatments with pioglytazone.

significantly change. GTN was also not significantly altered.

The change in FMD before and after administration of pioglitazone was not significantly correlated with the change in HbA_{1c} ($p = 0.314$) or fasting plasma glucose ($p = 0.717$). The increase in FMD, that is, the improvement in endothelial function, was significantly correlated with the decrease in SSPG ($r = -0.649$, $p < 0.05$).

4. Discussion

In this study we found that vascular endothelial dysfunction was associated with insulin resistance in type 2 diabetes. This result was supported by the effects of the insulin sensitizer, pioglitazone, which improved both endothelial dysfunction and insulin resistance in patients with type 2 diabetes.

The close association between insulin resistance and endothelial dysfunction is our main interest. In a study by Hogikyan et al. [3], insulin resistance as measured by the insulin sensitivity index (minimal model: S_I), was not found to be correlated with endothelial dysfunction in subjects with type 2 diabetes. They measured the forearm blood flow (FABF) using venous occlusion plethysmography and used the FABF response to acetylcholine as an index of endothelial function. The narrow range of S_I values among the subjects might have led to the lack of a relationship between S_I and endothelial dysfunction. In addition, the sensitivity of the techniques using plethysmography might have been low.

Balletshofer et al. [12] reported a significant association between endothelial dysfunction and insulin resistance, as measured by the glucose clamp method, in young normotensive and normoglycemic first-degree relatives of patients with type 2 diabetes. Therefore, this association was observed in a non-diabetic population at future risk of type 2 diabetes.

Insulin causes endothelium-derived nitric oxide (NO)-dependent vasodilation [13]. It is suggested that this insulin action occurs via the phosphatidylinositol 3-kinase and Akt pathway [14,15]. As for insulin action, phosphatidylinositol 3-kinase activation is critical for insulin-mediated glucose uptake into skeletal muscle [16]. Therefore, insulin resistance due to a systemic defect in the phosphatidylinositol 3-kinase pathway might cause a combined defect in insulin-mediated glucose uptake and insulin-mediated endothelial vasodilation.

Among the risk factors for atherosclerosis, insulin resistance was found to be the sole predictor of endothelium dependent vasodilation by multiple regression analysis in the present study. We observed no relationship between FMD and HbA_{1c}. Bagg et al. found that a short-term reduction of HbA_{1c} levels did not appear to affect endothelial function in patients with type 2 diabetes [17]. Furthermore, Mather et al. reported that insulin resistance was the sole predictor of endothelial dysfunction following metformin treatment in type 2 diabetes in stepwise multivariate analysis, and HbA_{1c} and glucose levels were not significant predictors of endothelial dysfunction [18].

Treatment with HMG-CoA inhibitors (statins) has been shown to improve endothelial dysfunction [19–21]. Therefore, statin treatment may have affected the relationship between FMD and risk factors in the present study. In 48 diabetic subjects, 5 were treated with pravastatin and one with simvastatin. We performed statistical analysis in 42 subjects without statin treatment. There was a significant inverse correlation between SSPG and FMD ($r = -0.538$, $p < 0.001$). A significant inverse correlation was observed between FMD and systolic blood pressure ($r = -0.330$, $p < 0.05$). No significant correlation was found between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.500$, $p = 0.0032$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age.

Smoking is associated with endothelial dysfunction [22,23]. Smoking might interfere in the relationship between FMD and risk factors. In 48 diabetic subjects, 13 were smokers in the present study. Statistical analysis was performed in 35 non-smokers. A significant correlation was found between SSPG and FMD ($r = -0.582$, $p < 0.001$). There was a significant inverse correlation between FMD and systolic blood pressure ($r = -0.357$, $p < 0.05$). No significant correlation was observed between FMD and HbA_{1c}, diabetic duration, diastolic blood pressure, total cholesterol, HDL cholesterol, triglyceride, age or BMI. On multiple regression analysis, FMD was independently related to SSPG (regression coefficient: $\beta = -0.591$, $p = 0.0019$) but not to systolic blood pressure, HbA_{1c}, total cholesterol, BMI or age. In the present study, FMD did not correlate with HOMA-IR. SSPG is a more sensitive marker to measure insulin sensitivity than HOMA-IR.

Endothelial dysfunction and insulin resistance were improved by pioglitazone treatment in the present study. SSPG, HbA_{1c} and fasting plasma glucose were decreased and other risk factors were not changed by the treatment. It was reported that hyperglycemia itself inhibits endothelial NO synthase activity [24] and causes endothelial dysfunction [25]. On the other hand,

insulin resistance was also associated with endothelial dysfunction in 48 subjects with type 2 diabetes in this study. The change in FMD before and after treatment with pioglitazone was not significantly correlated with the change in HbA_{1c} or fasting plasma glucose, and the increase in FMD was significantly correlated with the decrease in SSPG in this study. Because of the small number of subjects ($n = 10$), we cannot exclude the possibility that the decreased plasma glucose level improved endothelial dysfunction. The decrease in plasma glucose level might be associated with improved endothelial function if the pioglitazone study was performed with more cases. It can at least be said that insulin resistance is an important factor affecting endothelial function. As previously described, a similar study [18] found that treatment with metformin improved both endothelial function and insulin resistance, and the glucose level and HbA_{1c} were not significant predictors of endothelial dysfunction. Considering generally than the above-mentioned points, it is suggested that increased insulin sensitivity plays an important role in the improvement of endothelial function by pioglitazone treatment.

Pistrosch et al. [26] demonstrated that treatment with rosiglitazone, another PPARγ activator, ameliorated insulin resistance measured by glucose clamp method, and improved endothelial function determined by venous occlusion plethysmography in patients with recently diagnosed type 2 diabetes. They performed a double-blind cross-over trial and treated with rosiglitazone and nateglinide in random order. Glycemic control was comparable under rosiglitazone and nateglinide. Only rosiglitazone improved insulin resistance and endothelial function in the study. Thus, they also showed the relation between insulin sensitivity and endothelial function independent of glucose level in type 2 diabetes.

In conclusion, in the present study we demonstrated significant association between vascular endothelial dysfunction and insulin resistance in type 2 diabetes, and pioglitazone treatment improved both endothelial dysfunction and insulin resistance with a statistical link. These data support the concept of the important role of insulin resistance in the pathogenesis of endothelial dysfunction in type 2 diabetes mellitus.

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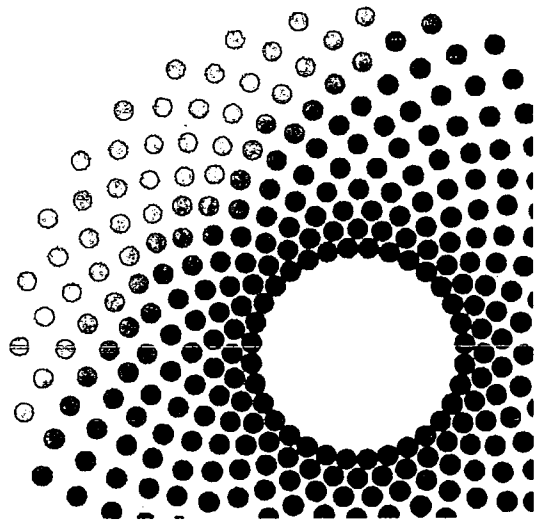

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Impaired flow-mediated vasodilatation and insulin resistance in type 2 diabetic patients with albuminuria

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ABSTRACT

An elevated urinary albumin excretion is associated with an increased risk of cardiovascular disease due to atherosclerosis, but the pathophysiological mechanism underlying this association is poorly understood. We studied 217 diabetic patients, that is, 121 normoalbuminuric patients, 71 microalbuminuric patients, and 25 macroalbuminuric patients. We evaluated flow-mediated dilatation of brachial artery (%FMD, one endothelial function marker associated with endogenous NO production), von Willebrand factor (vWF, endothelial activation marker), high-sensitive CRP (hsCRP, a low-grade inflammation marker), asymmetric dimethyl arginine (ADMA, an endogenous inhibitor of NO synthesis), and insulin sensitivity by steady-state plasma glucose method. %FMD was apparently decreased in microalbuminuric and macroalbuminuric patients compared with normoalbuminuric patients ($p < 0.001$). Moreover, %FMD was significantly correlated with the degree of albuminuria ($r = -0.38$, $p < 0.05$). On the other hand, vWF and hsCRP did not show significant difference between normoalbuminuric patients and microalbuminuric patients. In diabetic patients with macroalbuminuria, ADMA was significantly elevated compared to those with normoalbuminuria. Insulin sensitivity was significantly associated with urinary albumin excretion rate. These results suggested that endothelial dysfunction which may be due to impaired NO production and insulin resistance underlie the association between diabetic nephropathy and atherosclerosis in diabetic patients.

1. Introduction

Elevated urinary albumin excretion rate (UAER) is strongly associated with an increased risk of cardiovascular diseases, which is independent of conventional risk factors including hypertension, hyperlipidemia, and smoking, among individuals with and without type 2 diabetes [1,2]. This suggests that elevated UAER may be associated with atherosclerosis by the unidentified mechanism.

The endothelium plays a crucial role in the maintenance of vascular tone and structure, and endothelial dysfunction is a key feature of atherosclerosis. Nitric oxide (NO) is one of the important endothelium-derived vasoactive mediators. NO is involved in a wide variety of regulatory mechanisms of cardiovascular system, including vascular tone and vascular structure [3].

Flow-mediated endothelium-dependent vasodilatation (FMD) method is based on the endothelial stimulus of increased shear stress (the tangential force on the vessel wall exerted by flowing blood). Increased shear stress is caused by post-ischemic hyperemia and elicits a slow Ca^+-independent two to threefold increase in NO production [4,5]. Indeed,

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Celemajer et al. reported that flow mediate vasodilatation was mainly blocked by N-monomethyl-L-arginine (an inhibitor of endothelial NO synthetase) [6].

To clarify the contribution of impaired NO production in vascular endothelium to the association between atherosclerotic disease and diabetic nephropathy, we examined FMD by ultrasonography. In addition, we measured asymmetric dimethyl arginine (ADMA), an endogenous NO synthesis inhibitor [3]. Since low-grade inflammation is another key feature of the pathophysiology of atherosclerosis [7], we further examined high-sensitive CRP, which is an inflammation marker, to investigate whether this feature is involved in the association between atherosclerotic disease and diabetic nephropathy.

It has recently been indicated that microalbuminuria and atherosclerosis are closely associated with insulin resistance [8–10], implying that insulin resistance may underlie these pathophysiological conditions although the causative relationship remains unknown. In the present study, we further examined insulin sensitivity in the type 2 diabetic patients with different stage of albuminuria and analyzed the correlation between insulin sensitivity and FMD, to investigate whether elevated UAER and endothelial dysfunction may be associated with insulin resistance.

2. Methods

2.1. Study subjects

We studied 217 patients with type 2 diabetes who were <75 years of age. Patients with a current acute illness (including clinically significant infectious disease) were excluded from this study. Twenty-four-hour urine collections were performed for two consecutive days to determine the stage of diabetic nephropathy. Creatinine clearance (Ccr) was calculated from the 24-h urine sample and serum creatinine levels. The patients were divided into three groups according to the UAER, as follows: normoalbuminuria (UAER <30 mg/day), microalbuminuria (30 ≤ UAER < 100 mg/day) and macroalbuminuria (UAER ≥ 300 mg/day). To exclude diabetic patients with nondiabetic kidney disease, we excluded patients with hematuria or abnormal urinary sediments. This study was conducted with the approval of National Cardiovascular Center Trust Ethics Committee, and patients gave written informed consent before participation.

2.2. Brachial artery flow-mediated dilatation

Using ultrasonography, arterial endothelium and smooth muscle function were measured by examining brachial artery responses to endothelium-dependent and endothelium-independent stimuli. Ultrasoundsonographic measurements were carried out according to the method described by Celemajer et al. [6]. Brachial artery diameter was measured from B-mode ultrasound images using 10-MHz liner array transducer (ProSound SSD-5500; Aloka, Japan) while an ECG trace was simultaneously recorded. The right brachial artery was scanned in longitudinal sections 1–10 cm above elbow, after at least 15 min of rest in the supine position, the skin surface was marked and the arm was kept in the same position during the study.

Baseline measurements of the diameter were carried out. Endothelium-dependent vasodilatation (flow-mediated dilatation) was determined by scans during reactive hyperemia. A pneumatic cuff placed around the forearm was inflated to 220 mmHg and was deflated after 4.5 min. The diameter of the brachial artery was scanned and recorded after dilation. After 10 min rest, the second control scan of the diameter was recorded. Then, sublingual glyceryl trinitrate spray (300 μg) was administered and 3.5 min later a final scan of the diameter was recorded.

Measurements of the vessel diameter were taken from the anterior to posterior "m" line (interface between the media and adventitia) at end-diastole, coincident with the R wave on a continuously recorded ECG. The diameters at four cardiac cycles were measured for each scan, and these results were averaged. Determinations of the FMD were carried out 45–60 s after the cuff release to measure a maximal diameter. Vasodilatation by reactive hyperemia or glyceryl trinitrate (NTG) was expressed as the percent change in diameter compared with the baseline values.

2.3. Insulin sensitivity test

Glucose utilization in response to insulin was evaluated with a newly modified steady-state plasma glucose (SSPG) method with octreotide acetate (Sandostatin; Novartis) after an overnight fasting period of 12 h [11]. Sandostatin (9.8-pmol bolus followed by a constant infusion of 73.5 pmol/h) and Humulin R insulin (45 pmol/kg bolus followed by a constant infusion at a rate of 4.62 pmol/(kg min); Eli Lilly) were infused intravenously for 120 min. Glucose in a final 12% solution containing KCl (0.5 μmol/(kg min)) was infused at a rate of 0.033 mmol/(kg min) (6 mg/(kg min)) through an antecubital vein via a constant infusion pump. Blood samples were drawn routinely at 0 and 120 min (9:00 and 11:00 a.m.) for the determination of glucose, insulin, and lipids. The value of glucose at 120 min (SSPG) was used as a marker of insulin sensitivity to glucose utilization. High SSPG levels showed peripheral insulin resistance.

Another marker of insulin resistance (IR) was estimated by calculating homeostasis model assessment (HOMA-IR) index ((fasting serum insulin (μU/ml) × fasting plasma glucose (mmol/l))/22.5) [12].

2.4. Measurement of vWF, hsCRP, and ADMA

vWF was determined in citrated plasma using a homemade enzyme-linked immunosorbent assay. Data are given as the percentage of pooled human plasma (set at 100%). Serum hsCRP concentration was determined by latex nephelometry method (SRL, Tokyo, Japan). Serum ADMA concentration was determined by high-performance liquid chromatography method (SRL, Tokyo, Japan).

2.5. Statistical analysis

Values are expressed as means ± S.D. Statistical analysis was performed by use of ANOVA followed by Scheffes' test. The

Table 1 – Characteristics of diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
n	121	71	25
Age (years)	62 ± 9	65 ± 8	66 ± 7
Men/women	76/45	34/37	12/13
Duration of diabetes (years)	12 ± 8	14 ± 8	18 ± 8*
BMI (kg/m^2)	25.0 ± 3.7	25.1 ± 3.7	25.1 ± 3.9
SBP (mmHg)	128 ± 13	133 ± 15	141 ± 19*
DBP (mmHg)	74 ± 10	73 ± 9	76 ± 10
FBS (mmol/l)	7.4 ± 1.4	7.5 ± 1.5	7.5 ± 1.9
HbA1c (%)	8.3 ± 1.5	8.9 ± 1.7*	8.8 ± 1.4
HOMA-IR	1.62 ± 0.98	1.71 ± 2.06	2.29 ± 1.47
Total cholesteroyl (mmol/l)	4.86 ± 0.90	4.86 ± 0.90	4.73 ± 0.75
Serum creatinine (μmol/l)	70 ± 20	60 ± 20	110 ± 40
Urinary albumin (mg/day)	10 ± 7	85 ± 79**	583 ± 576**
Creatinine clearance (ml/s)	1.43 ± 0.52	1.50 ± 0.63	0.73 ± 0.43**
ACEI or ARB (yes/no)	36/85	24/47	11/14*
Statin (yes/no)	45/76	25/46	10/15
Current smoker (yes/no)	11/110	7/64	6/19

*$p < 0.05$, **$p < 0.01$ vs. normoalbuminuria, mean ± S.D.

strength of correlation between variables was tested by linear correlation and multiple regression analysis. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Patients characteristics

Table 1 shows the clinical characteristics of three groups. There was no significant difference in age, gender, BMI, FBS and total cholesterol among the three groups. HbA1c of diabetic patients with microalbuminuric patients was significantly higher than normoalbuminuric patients. Systolic blood pressure of macroalbuminuric patients was significantly higher than normo- and micro-albuminuric patients. Creatinine clearance was significantly decreased in macroalbuminuric patients compared with normo- and micro-albuminuric patients. There is no significant difference in rate of patients taking ACE/ARB between normo- and micro-albuminuric patients whereas the rate of patients taking ACE/ARB of macroalbuminuric patients were significantly large compared with other two groups. On the other hand, there is no significant difference in rate of patients taking statin among three groups.

3.2. %FMD of diabetic patients

We studied the endothelial function by FMD using brachial artery echography. %FMD (Δhyperemia) of diabetic patients with microalbuminuria (4.5 ± 3.7%) and macroalbuminuria (4.2 ± 2.4%) was apparently decreased compared with those of diabetic patients with normoalbuminuria (6.6 ± 3.7%) (Fig. 1A). Moreover, %FMD was significantly correlated with UAER in normo- and micro-albuminuric patients independent of age, HbA1c, and systolic blood pressure by multiple regression analysis ($r = -0.38$, $p < 0.05$) (Fig. 2). Dilatation of brachial artery by NTG (ΔNTG) showed no difference among three groups (Fig. 1B).

3.3. vWF, hsCRP, and ADMA of diabetic patients

We studied other atherosclerotic markers, that is, vWF, hsCRP, and ADMA. There was no significant difference of the levels of vWF and hsCRP between normoalbuminuric and microalbu-

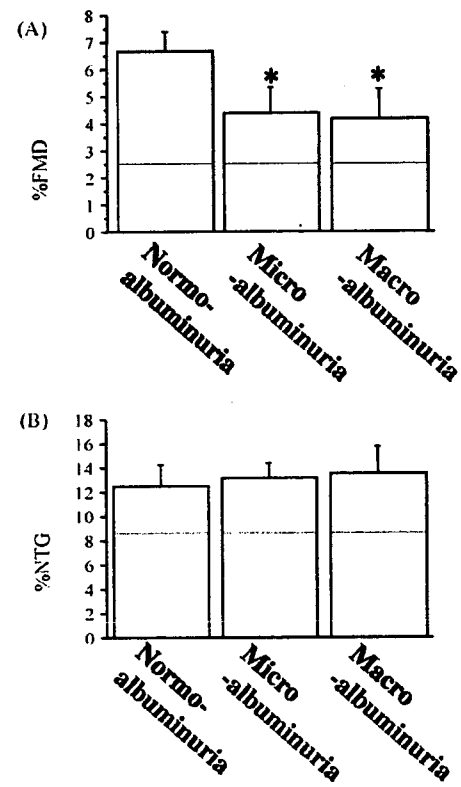

Fig. 1 – %FMD (A) and %NTG (B) in diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria. Each value means (means ± S.D.), *$p < 0.001$.

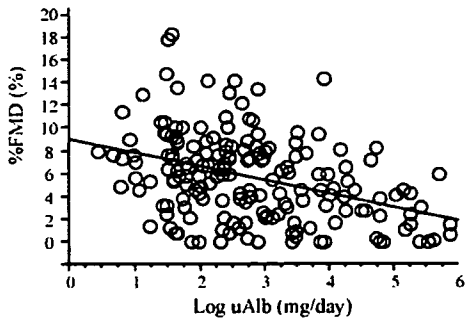

Fig. 2 – Correlation between degree of UAE and %FMD in normo- and micro-albuminuric diabetic patients. There was a significant correlation between both variables ($r = -0.38$, $p < 0.05$, $n = 192$).

minuric patients (Table 2). Although the levels of ADMA in microalbuminuric patients did not show significant difference compared with normoalbuminuric patients (Table 2), the levels of ADMA in macroalbuminuric patients were significantly elevated compared with normoalbuminuric patients (Table 2).

3.4. *Insulin sensitivity of diabetic patients*

We studied the insulin sensitivity by SSPG method. The levels of SSPG had weak but significant correlation with both %FMD ($r = -0.175$, $p < 0.05$) and UAER ($r = 0.181$, $p < 0.05$) independent of age, HbA1c, and systolic blood pressure (Fig. 3A, B).

4. Discussions

There were two main findings from this investigation in type 2 diabetic patients. First, diabetic micro- and macro-albuminuric patients showed significant reduction of %FMD compared with normoalbuminuric patients. This finding suggests that the endothelial dysfunction may account for the association between atherosclerosis and albuminuria in diabetic patients. Second, the level of SSPG was significantly associated with both UAER and %FMD. This finding suggests that insulin resistance may play a role in both atherosclerosis and nephropathy in type 2 diabetic patients.

In diabetic patients, %FMD is decreased compared with healthy control [13,14]. These reports indicated that diabetes mellitus is associated with endothelial dysfunction due to

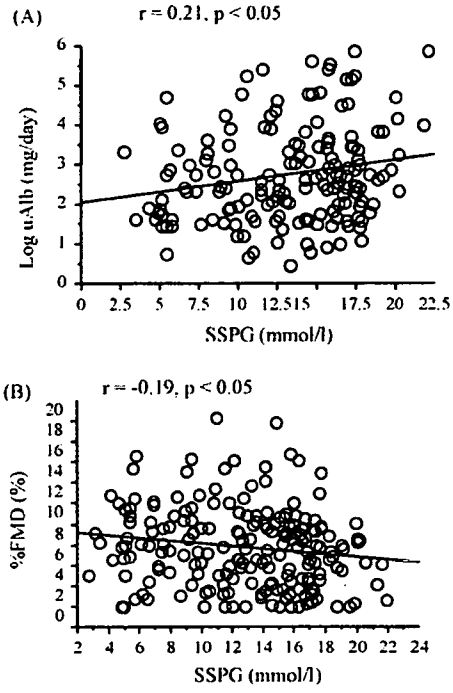

Fig. 3 – Correlation between SSPG and UAE (A), and correlation between SSPG and %FMD (B) in normo- and micro-albuminuric patients.

impaired NO production. However the involvement of endothelial dysfunction in diabetic nephropathy has been unclarified. We demonstrated that microalbuminuric and macroalbuminuric patients showed significant decreased %FMD compared with normoalbuminuric patients. In contrast, there was no significant difference of vWF between normoalbuminuric patients and microalbuminuric patients. vWF is a product of vascular endothelial cell, and induces coagulation and platelet aggregation [15]. These findings suggest that endothelial dysfunction due to impaired NO production is specifically induced in micro- and macroalbuminuric patients. One recent report showed that coronary endothelium-dependent dilatation was impaired in a rat model of spontaneous albuminuria [16] supporting this hypothesis. It has been reported that renal NO production was decreased in rodent diabetic model [17]. This report suggests that decrease of NO production may play a role in the

Table 2 – Parameters of atherosclerosis in diabetic patients with normoalbuminuria, microalbuminuria, and overt nephropathy

Parameter	Stage of nephropathy		
	Normoalbuminuria	Microalbuminuria	Macroalbuminuria
von Willebrand factor (%)	147 ± 44	146 ± 44	143 ± 41
High-sensitive CRP (ng/ml)	976 ± 1401	951 ± 1110	1113 ± 1187
ADMA (nmol/ml)	0.45 ± 0.06	0.47 ± 0.07	0.55 ± 0.11*

*$p < 0.001$ vs. normoalbuminuria, mean ± S.D.

progression of diabetic nephropathy as well as atherosclerosis. We investigated serum ADMA levels in diabetic patients. There was no significant difference of ADMA levels between normo- and micro-albuminuric patients, suggesting that the reduction of %FMD in microalbuminuric patients might not be resulted from the elevation of ADMA. However, in macroalbuminuric patients, ADMA level was significantly higher than normoalbuminuric patients. Vallance et al. reported that the level of ADMA was elevated in patients with chronic renal failure and suggested the involvement of this in coronary artery disease [18]. They indicate that the elevation of ADMA might be associated with atherosclerosis in patients with chronic renal disease [18]. Thus, this finding suggests that the elevation of ADMA might be associated with atherosclerotic change in diabetic patients with macroalbuminuria.

An association between chronic low-grade inflammation and development of atherosclerotic disease has been observed in basic and clinical studies [7,19–21]. Furthermore, diabetic patients have higher CRP levels than normal subjects, suggesting that chronic inflammation may contribute diabetic atherosclerotic complication [22]. An association between micro- and macro-albuminuria and inflammation has also been reported [23,24]. However, several other studies showed that inflammatory molecules were not associated with micro- and macro-albuminuria [25–27]. Thus the knowledge of this association is still controversial. Also we could not demonstrate the association between CRP and development of microalbuminuria in this study. Our data suggested that chronic low-grade inflammation might not be involved in the association between atherosclerosis and microalbuminuria. However, since this study was performed by cross-sectional analysis and other inflammatory marker was not measured, further study is necessary for demonstrating this hypothesis.

Insulin resistance has been reported to play an important role in the development and progression of atherosclerotic coronary disease [8,9]. Recently the association between insulin resistance and microalbuminuria was also reported [10]. Nakamura et al. demonstrated that administration of pioglitazone to diabetic patients attenuated UAER [28]. In this study, we showed that both the UAER and %FMD were significantly correlated to the level of SSPG. These findings suggest that insulin resistance may be involved in both the elevated urinary albumin excretion and endothelial dysfunction due to impaired NO production. However, HOMA-IR, another insulin sensitivity marker which reflects insulin sensitivity in both the liver and the periphery, did not show significant difference among three groups, suggesting that particularly peripheral insulin resistance may be important for the pathogenesis of atherosclerosis and diabetic nephropathy.

In summary, we showed that %FMD of micro- and macro-albuminuric patients was decreased compared with those of normoalbuminuric patients, without showing significant difference in other various atherosclerotic markers. Furthermore, the level of SSPG was significantly correlated to UAER and %FMD. These findings suggest that endothelial dysfunction which may be due to impaired NO production underlies the mechanism of association between elevated urinary albumin excretion and atherosclerosis in diabetic patients, and that peripheral insulin resistance might be possibly involved in both diabetic nephropathy and atherosclerosis.

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Circulating CD34-Positive Cell Number Is Associated With Brain Natriuretic Peptide Level in Type 2 Diabetic Patients

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Patients with type 2 diabetes often suffer from asymptomatic left ventricular (LV) injury, including increased LV mass, without apparent myocardial ischemia. The mechanisms underlying diabetic LV injury remain unclear; however, it has been suggested that endothelial dysfunction plays a role. Accumulating evidence indicates that bone marrow–derived endothelial progenitor cells (EPCs) contribute to neovascularization of ischemic tissue and endothelialization of denuded endothelium. Recent studies have shown that circulating bone marrow–derived immature cells, including $CD34^+$ cells, contribute to the maintenance of the vasculature, both as a pool of EPCs and as the source of growth/angiogenesis factors (1). We hypothesized that circulating $CD34^+$ cells might be associated with LV dysfunction in patients with type 2 diabetes. Therefore, we studied the correlation between circulating $CD34^+$ cell levels and plasma brain natriuretic peptide (BNP) levels, an LV dysfunction marker, in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

The institutional review board of the National Cardiovascular Center approved this study, and all subjects provided informed consent. We examined 26 patients with type 2 diabetes (12 men and 14 women, duration of diabetes 16.1 ± 10.7 years) who were over 60 years of age (70.5 ± 6.4 years). Statin was given to nine subjects. ACE inhibitor or angiotensin receptor blocker was given to nine subjects, and thiazolidinedione was given to two subjects. Subjects were excluded from the study if they had known cardiovascular disease or chronic renal failure (defined as serum creatinine ≥180 μmol/l). No study subject showed hypokinesis by echocardiography or electrocardiogram change, indicating myocardial ischemia. Systolic (SBP) and diastolic (DBP) blood pressure and anthropometric parameters were determined. Blood samples were taken after 12-h fasting to measure circulating $CD34^+$ cells, plasma BNP, fasting plasma glucose (FPG), and A1C. Circulating $CD34^+$ cells were quantified by flow cytometry according to the manufacturer's protocol (ProCOUNT; Becton Dickinson Biosciences) as previously reported (2). BNP was quantified by enzyme immunoassay (Tohso, Tokyo, Japan). We further examined LV fractional shortening (LVFS), LV mass index (LVMI) (3), and peak flow velocity of the early filling wave (E), the late filling wave (A), and the E/A-wave ratio (E/A) by echocardiography. All echocardiograms were performed by several expert physicians who were blinded to $CD34^+$ cell level.

All statistical analyses were performed using JMP version 5.1.1 software (SAS Institute). Data are expressed as means ± SD. Comparisons of number of $CD34^+$ cells by sex were made using the two-tailed unpaired *t* test. Correlations between number of $CD34^+$ cells and clinical parameters were assessed by univariate liner regression analysis and multiple regression analysis. LVMI and plasma BNP concentrations were analyzed after logarithmic transformation.

RESULTS

FPG levels, A1C levels, and BMIs in the study subjects were measured to be 9.5 ± 2.6 mmol/l, 9.2 ± 1.8%, and 26.4 ± 4.3 kg/m^2, respectively. A total of 88% of the patients had hypertension (SBP 142 ± 18 mmHg, DBP 75.7 ± 13.5 mmHg). Plasma BNP levels were measured to be 95 ± 319 pg/ml. Although it has been reported that the level of BNP ≥100 pg/ml has a sensitivity of 90% of diagnosing congestive heart failure (CHF) in patients with CHF symptoms (4), none of the subjects in this study, including subjects with ≥100 pg/ml of BNP, showed symptoms of CHF. The level of circulating $CD34^+$ cells was measured to be 0.76 ± 0.39 cells/μl, and there was no significant difference between sexes. The range of LVMI was 73.3–340.2, and 11 subjects applied to the definition of LV hypertrophy (LVMI ≤131 in men and ≤100 in women) (3).

Plasma BNP levels had a significant inverse correlation with the number of circulating $CD34^+$ cells (Fig. 1*A*), whereas FPG, A1C, BMI, SBP, DBP, and age showed no significant correlations. There was a significant correlation between the number of circulating $CD34^+$ cells and LVMI by echocardiography (Fig. 1*B*). LVFS and E/A were not associated with circulating $CD34^+$ cell numbers (LVFS $r = -0.07$, $P = 0.72$; E/A $r = -0.11$, $P = 0.59$). There was also a significant correlation between BNP levels and LVMI ($r = 0.59$, $P = 0.001$).

In multiple regression analysis, the

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Abbreviations: BNP, brain natriuretic peptide; CHF, congestive heart failure; DBP, diastolic blood pressure; EPC, endothelial progenitor cell; FPG, fasting plasma glucose; LV, left ventricular; LVFS, LV fractional shortening; LVMI, LV mass index; SBP, systolic blood pressure.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

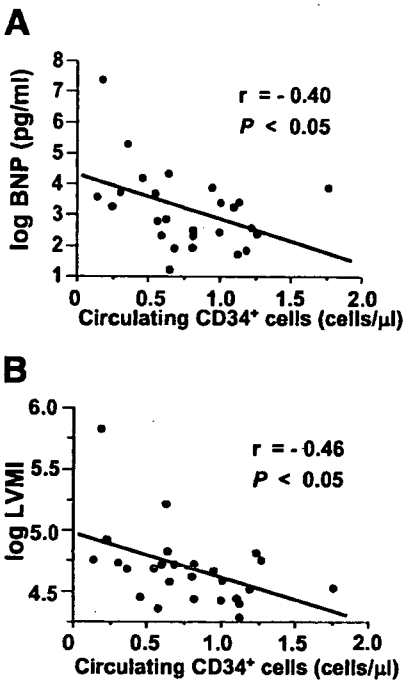

Figure 1—*Correlation between CD34$^+$ cell numbers and plasma BNP levels (A) and correlation between CD34$^+$ cell numbers and LVMI (B) in type 2 diabetic patients* (n = 26).

level of CD34$^+$ cells was an independent correlate of both BNP ($\beta = -1.64$, $P = 0.017$) and LVMI ($\beta = -0.337$, $P = 0.031$) in the model including age, A1C, SBP, BMI, and medication (ACE inhibitor/angiotensin receptor blocker, statin, and thiazolidinedione).

CONCLUSIONS — In this study, circulating CD34$^+$ cell number was found to significantly correlate with plasma BNP level, a marker of LV dysfunction. To the best of our knowledge, this is the first report that circulating bone marrow–derived cells are associated with diabetic LV abnormality. Circulating CD34$^+$ cell numbers also significantly correlated with LVMI, whereas they did not correlate with LVFS (an LV systolic function marker) or E/A (an LV diastolic function marker). LV hypertrophy is a well-known predictor of cardiovascular events independent of coronary artery disease. The Framingham Heart Study identified an association between diabetes and increased LV wall thickness and mass (5). Although the precise mechanisms underlying the association between diabetes and LV hypertrophy remain unknown, our results suggest that reduced circulating CD34$^+$ cell numbers may be involved in the progression of LV hypertrophy in diabetic patients. However, further investigations are necessary to demonstrate this hypothesis.

We measured the level of CD34$^+$ cells in this study but not the levels of circulating CD34$^+$/kinase insert domain receptor (KDR)$^+$ cells that are regarded as EPCs. Circulating CD34$^+$ cell levels are associated with ischemic stroke (6), and administration of CD34$^+$ cells ameliorates cerebral ischemia in mice (7). This indicates that CD34$^+$ cells may be involved in cardiovascular disease. Indeed, another recent report indicated that levels of circulating CD34$^+$ cells are more strongly correlated with cardiovascular risk than levels of EPCs (8). Therefore, our results suggest that measurement of CD34$^+$ cells may provide an indicator for diabetic LV hypertrophy.

Our study had several limitations. First, the study was performed only by cross-sectional analysis; therefore, a prospective study is needed to clarify whether circulating CD34$^+$ cell numbers predict LV injury in diabetic patients. Second, although systemic blood pressure did not significantly associate with CD34$^+$ cell numbers, further investigation of normotensive diabetic patients is needed to exclude the possible effects of hypertension on circulating CD34$^+$ cell numbers, as most of the subjects in this study were hypertensive. Despite this caveat, these results may be of practical use in elderly patients with type 2 diabetes, as hypertension is a very common comorbid condition in this population.

In conclusion, reduced circulating CD34$^+$ cell numbers are significantly associated with plasma BNP concentration and LVMI in elderly patients with type 2 diabetes. These results suggest that decreased circulating CD34$^+$ cells may be involved in LV hypertrophy and that measurement of circulating CD34$^+$ cell numbers may be useful for the identification of diabetic patients at high risk of LV injury.

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ORIGINAL ARTICLE

Reverse white-coat effect as an independent risk for left ventricular concentric hypertrophy in patients with treated essential hypertension

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Recent studies have shown that the converse phenomenon of white-coat hypertension called 'reverse white-coat hypertension' or 'masked hypertension' is associated with poor cardiovascular prognosis. We assessed the hypothesis that this phenomenon may specifically influence left ventricular (LV) structure in treated hypertensive patients. A total of 272 outpatients (mean age, 65 years) with chronically treated essential hypertension and without remarkable white-coat effect were enrolled. Patients were classified into two groups according to office and daytime ambulatory systolic blood pressure (SBP); that is subjects without (Group 1: office SBP $\geqslant$daytime SBP, $n=149$) and with reverse white-coat effect (Group 2: office SBP<daytime SBP, $n=123$). LV mass index and relative wall thickness were echocardiographically determined. In all subjects, LV mass index and relative wall thickness were positively correlated with daytime and 24-h SBP, but not with office SBP. In addition, these two indices were inversely correlated with office – daytime SBP difference. LV mass index (136 ± 31 and 115 ± 28 g/m^2, mean$\pm$s.d.) and relative wall thickness (0.49 ± 0.09 and 0.46 ± 0.07) were significantly greater in Group 2 than in Group 1. As for LV geometric patterns, Group 2 had a significantly higher rate of concentric hypertrophy compared with Group 1 (48 and 28%). Multivariate analyses revealed that the presence of reverse white-coat effect was a predictor for LV concentric hypertrophy, independent of age, sex, hypertension duration, antihypertensive treatment and ambulatory blood pressure levels. Our findings demonstrate that reverse white-coat effect is an independent risk factor for LV hypertrophy, especially concentric hypertrophy, in treated hypertensive patients.

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Introduction

Ambulatory blood pressure (BP) is an important determinant of target organ damage and a significant predictor for cardiovascular morbidity and mortality in hypertensive patients.[1–6] There is often a discrepancy between office and ambulatory BPs, such as white-coat hypertension, a normal ambulatory but elevated office BP. On the other hand, the converse phenomenon of white-coat hypertension called 'reverse white-coat hypertension' or 'masked hypertension', that is, a high ambulatory but normal (or well-controlled) office BP, has received little attention.[7] Whereas, some studies have revealed that the proportion of subjects with reverse white-coat condition reaches 20–40% of the general population and hypertensives.[8,9] In treated hypertensive patients with this phenomenon, particularly, the chance of active and sufficient antihypertensive treatment may be lost by an apparent well-controlled BP in the office. Recent studies suggested that an elevated ambulatory or home BP despite a well-controlled office BP is associated with poor cardiovascular prognosis in treated hypertensive patients.[10,11] However, it remains unclear what mechanism is involved in the association of reverse white-coat phenomenon with cardiovascular prognosis.

Left ventricular hypertrophy (LVH), which is a common cardiac consequence of hypertension, is well known to be an independent risk factor for cardiovascular complications and death.[12,13] In addition, left ventricular (LV) morphologic

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alteration in hypertensive patients is not uniform, and concentric hypertrophy among various LV geometric patterns is shown to be most closely related to poor cardiovascular prognosis.[13]

Thus, we hypothesized that the presence of reverse white-coat effect may promote LV hypertrophy, especially concentric hypertrophy, in treated hypertension. To assess the hypothesis, the present study investigated the influence of reverse white-coat effect on LV mass and geometry in treated hypertensive patients.

Methods

Subjects

From consecutive patients with essential hypertension who were chronically treated and underwent a 24-h ambulatory BP monitoring at an outpatient clinic of our hospital between May 2000 and December 2003, 272 subjects (142 men and 130 women; mean age, 65 years) in whom satisfactory echocardiographic data were simultaneously obtained were enrolled in the present study. Patients with secondary hypertension, stroke, ischaemic heart disease including myocardial infarction, congestive heart failure, renal failure (serum creatinine $\geqslant 160\,\mu$mol/l) or poorly controlled (haemoglobin A1c $\geqslant 8.0\%$) or insulin-treated diabetes mellitus were excluded from this study. Individuals with a remarkable white-coat effect (described below) were also excluded. Diabetes mellitus was diagnosed according to the American Diabetes Association criteria, such as a fasting plasma glucose of $\geqslant 7.0$ mmol/l and/or a plasma glucose level at 2 h after a 75-g oral glucose load of $\geqslant 11.1$ mmol/l, or when medication was taken for treatment of hyperglycaemia. A diagnosis of hyperlipidemia required a serum total cholesterol level of $\geqslant 5.69$ mmol/l and/or a serum triglyceride level of $\geqslant 1.69$ mmol/l or the use of lipid-lowering drugs, according to the Japan Atherosclerosis Society guidelines.[14]

All patients had taken antihypertensive drugs for at least 1 year (average, 12 years). One hundred and ninety-five patients (72%) were treated with Ca channel blockers, 140 (51%) with renin angiotensin system inhibitors (i.e., angiotensin II receptor blockers and angiotensin converting enzyme inhibitors), 82 (30%) with β-blockers, 53 (19%) with diuretics and 29 (11%) with other classes of agents. All subjects gave their informed consent to participate in the present study. All procedures of the present study were carried out in accordance with institutional and national ethical guidelines for human studies.

Measurement of BP

In each visit, office BP was measured twice by a physician in a hospital outpatient clinic with the patient in a sitting position after over 20 min of rest, using an appropriate-size cuff on the left arm and mercury sphygmomanometer. The first and fifth Korotkoff sounds were used to identify systolic and diastolic values, respectively, and measurements were taken to the nearest 2 mm Hg. Office BP was determined by averaging six measurements taken on three separate occasions during a 3-month period.

In the same study period, all subjects underwent 24-h ambulatory BP monitoring. BP was measured every 30 min during the day and night by the oscillometric method using an automatic monitoring device (TM-2421, A&D Co Ltd, Tokyo, Japan).[15] The accuracy and performance of this device have been demonstrated previously.[16] The patients were instructed to carry on with their normal daily activities during measurements and note their activity and location in a diary. According to the diary, daytime and night time were determined as the waking and sleeping periods of the patient, respectively, and mean values of daytime, night time and 24-h BP (systolic and diastolic) were calculated. Nocturnal BP dipping was determined as $100 \times$ (daytime BP$-$night time BP)/daytime BP.

In the present study, all subjects were classified into two groups by the difference between office and daytime ambulatory systolic BP levels; that is, subjects without reverse white-coat effect (Group 1: office systolic BP $\geqslant$ daytime systolic BP, and office systolic BP$-$daytime systolic BP <20 mm Hg) and with reverse white-coat effect (Group 2: office systolic BP $<$ daytime systolic BP). Subjects with a remarkable white-coat effect (office systolic BP$-$daytime systolic BP $\geqslant 20$ mm Hg) were excluded from the study.

Echocardiography

A comprehensive 2-dimensional and M-mode echocardiography was performed using a cardiac ultrasound unit (Sonos 5500, Philips Medical Systems, Andover, MA, USA) as described previously.[17] Echocardiographic parameters were measured by the consensus of two experienced investigators who were blinded to the clinical data including office and ambulatory BP of the subjects. Interventricular septal thickness (IVSTd), posterior wall thickness (PWTd), LV diameter at end-diastole (LVDd), and LV diameter at end-systole (LVDs) were measured according to the American Society of Echocardiography recommendations.[18,19] Fractional shortening was calculated as $100 \times$ (LVDd$-$LVDs)/LVDd. Relative wall thickness (RWT) was calculated as (IVSTd + PWTd)/LVDd. LV mass was estimated using the formula validated by Devereux and Reichek[20]: LV mass (g) $= 1.04 \times \{$(IVSTd + PWTd + LVDd)^3 $-$ LVDd$^3\} - 13.6$. LV mass was normalized for body surface area and expressed as the LV mass index (LVMI). LVH was defined as a LVMI of $\geqslant 125$ g/m^2 in men and 110 g/m^2 in women.[21] The intra-observer and inter-observer coefficients of variation of LVMI were 6.7 and 9.8%, respectively.