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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 ($30 \leq$ 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-

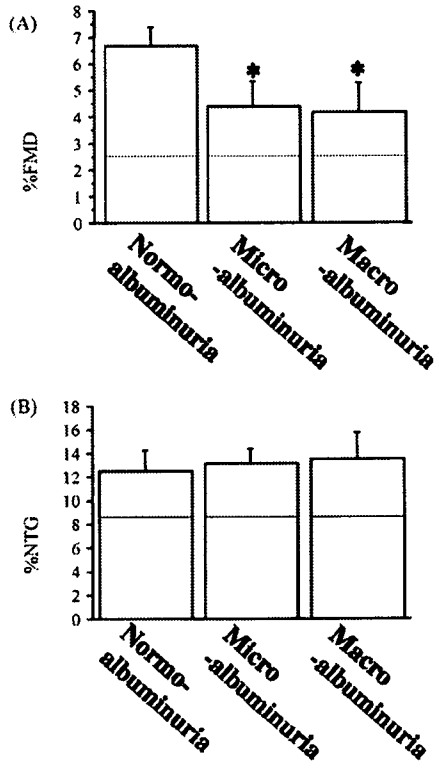

Fig. 1 – %FMD (A) and %NTG (B) in diabetic patients with normoalbuminuria, microalbuminuria and macroalbuminuria. Each value means (means ± S.D.), *$p < 0.001$.

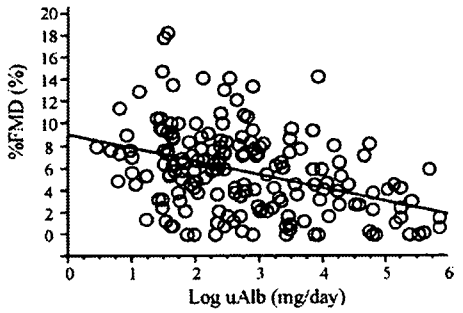

Fig. 2 – Correlation between degree of UAE and %FMD in normo- and micro-albuminuric diabetic patients. There was a significant correlation between both variables ($r = -0.38$, $p < 0.05$, $n = 192$).

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).

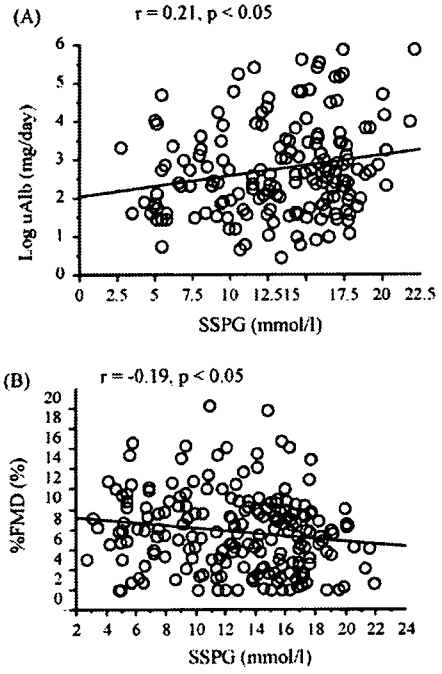

Fig. 3 – Correlation between SSPG and UAE (A), and correlation between SSPG and %FMD (B) in normo- and micro-albuminuric patients.

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 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.

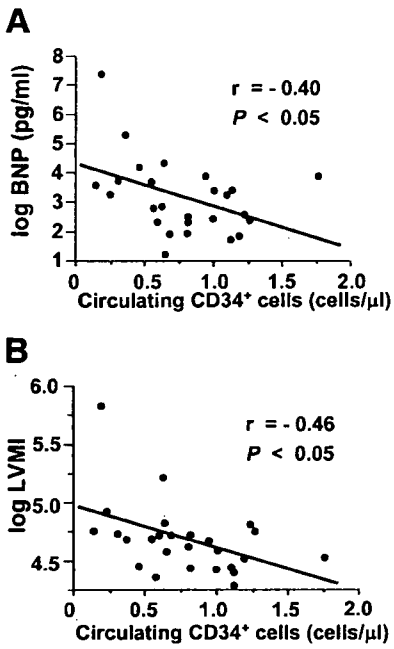

Figure 1—*Correlation between CD34$^+$ cell numbers and plasma BNP levels (A) and correlation between CD34$^+$ cell numbers and LVMI (B) in type 2 diabetic patients (n = 26).*

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

Association of single nucleotide polymorphisms in endothelin family genes with the progression of atherosclerosis in patients with essential hypertension

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Endothelin-1 (ET-1) is a potent vasoconstrictive peptide and its activity is mediated by the receptors ET type A (EDNRA) and ET type B (EDNRB). Although ET-1 is thought to play an important role in the development of atherosclerosis, it remains unclear whether polymorphisms of ET-1 family genes, including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*), are associated with the progression of atherosclerosis. We investigated the relationship between 11 single nucleotide polymorphisms (SNPs) of ET-1 family genes (including three in *EDN1*, one in *EDNRA*, two in *EDNRB*, four in *ECE1* and one in *ECE2*) and atherosclerotic changes assessed using pulse wave velocity (PWV) and carotid ultrasonography in 630 patients with essential hypertension (EHT). In male subjects, we found significant differences in brachial-ankle PWV (baPWV) in additive and recessive models in *EDNRB*-rs5351 after Bonferroni correction. Also in male subjects, there were significant differences in mean intima-media thickness (IMT) in additive and recessive models in *EDNRA*-rs5333 after Bonferroni correction. We found no significant correlation between any SNPs in the ET family genes and baPWV, IMT and Plaque score (PS) in female subjects. Furthermore, after multiple logistic regression analysis, only *EDNRB*-rs5351 indicated as an independent risk of atherosclerosis in male hypertensive subjects. Of the endothelin-related genes, *EDNRB*-rs5351 was the most susceptible SNP associated with atherosclerosis in male hypertensives, and the genetic background may be involved in the progression of atherosclerosis in EHT patients.
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Keywords: endothelin 1 (ET-1) family genes; single nucleotide polymorphisms (SNPs); atherosclerosis

Introduction

Endothelin-1 (ET-1) is a potent vasoconstrictive peptide produced primarily by vascular endothelial cells and appearing in many other organs.[1] ET-1 is thought to play an important role in the development of atherosclerosis through endothelial dysfunction and the proliferation of vascular smooth muscle cells (VSMCs). ET-1 may be a marker for arterial vascular disease; Lerman *et al.*[2] showed a significant correlation between plasma endothelin levels and the number of vascular disease sites. Some reports have linked plasma levels of ET-1 to hypertension, while others have argued against this relationship. Hirai *et al.*[3] suggested that high ET-1 levels are not related to hypertension, but rather to subclinical renal dysfunction and smoking. The expression of ET-1 is mediated by the activation of specific receptors: ET type A (EDNRA) and ET type B (EDNRB). The former is the predominant ET receptor on VSMCs, and signalling via EDNRA causes long-lasting vasoconstriction.[4,5] EDNRB is located primarily on endothelial cells and its signalling promotes the formation of nitric oxide, as well as the clearance and reuptake of ET-1.[6–9] Endogenous ET-1, which acts via EDNRA, increases resistance-vessel tone in subjects with hypertension to a level greater than that in smokers and in subjects with hypercholesterolemia.[10] Plasma ET-1

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concentrations can be reduced by resistance training and aerobic exercise.[11]

Pulse wave velocity (PWV) is generally recognized as a surrogate marker for atherosclerosis.[12] Using sheep, McEniery *et al.*[13] showed that endogenous ET-1 production regulates large artery PWV *in vivo*. They also revealed that exogenous ET-1 increases PWV and that this increase can be blocked by ET type A receptor blockers. Vuurmans *et al.*[14] examined whether ET-1 increases central aortic systolic blood pressure, pulse pressure and PWV in healthy men, and the effect of ET-1 is prevented by ET-1 receptor blockers.

It remains unclear, however, whether gene polymorphisms of the ET-1 family (including the ET-1 gene (*EDN1*), *EDNRA*, *EDNRB* and the genes for endothelin converting enzymes 1 and 2 (*ECE1* and *ECE2*) are associated with the progression of atherosclerosis. Therefore, we investigated the relationship between single nucleotide polymorphisms (SNPs) of ET-1 family genes and atherosclerotic changes assessed by PWV and carotid echo ultrasonography in patients with essential hypertension (EHT).

Materials and methods

Subjects

This study included 630 outpatients (340 men and 290 women) with EHT at the Division of Hypertension and Nephrology of the National Cardiovascular Centre (NCVC). All subjects provided written informed consent and the protocol was approved by the ethics committee of NCVC. Hypertension was defined as a systolic blood pressure (SBP) of 140 mm Hg or greater and/or a diastolic blood pressure (DBP) of 90 mm Hg or greater, or the current use of antihypertensive medication. The blood pressure used was the average of at least three measurements made during each visit. We also measured brachial-ankle PWV (baPWV) using Form ABI (Colin Medical Technology) and examined carotid arteries using a commercially available ultrasound system (SSA-390A; Toshiba Medical, Japan).[4] We measured the mean intima-media thickness (IMT) and maximum-IMT (max-IMT) of common carotid arteries and the sum of the plaque score (PS) of bilateral common and internal carotid arteries, as reported previously.[15] Blood samples were also taken at the clinic, and diabetes mellitus was defined as a fasting blood sugar level greater than 126 mg/dl, an HbA_{1c} level greater than 6.5%, or the use of anti-hyperglycemic medications. Hyperlipidemia was defined as a total-cholesterol concentration of 220 mg/dl or greater, a triglyceride (TG) concentration of 150 mg/dl or greater, or the use of lipid-lowering medication at the time of the first examination. Subjects who had ankle-brachial indices (ABI) lower than 0.9 were excluded because their baPWV readings were unreliable.

Table 1 The entire coding region of the endothelin-1 gene

		Allele 1/Allele 2			*Allele 1*		*Allele 2*		*Allele frequency*			
Gene name	*Locus*	*SNPs*	*Aa info.*	*Region*	*Homo*	*Hetero*	*Homo*	*Total*	*Allele 1*	*Allele 2*	*Flanking sequence*	*dbSNP ID*
EDN1	6p24-p23	10bp del.(−173)		promoter	47	1	0	48	0.990	0.010	AGGTTTAGCAAA[GGTCTCTAAT/-]GGGTATTTTCTT	
		A201- (4A/3A)	5′-UTR	exon1	1	8	39	48	0.104	0.896	TTTCTCCCCGTT[A/-]AAAGGGCACTTG	
		G2087A	Gly36Arg	exon2	47	1	0	48	0.990	0.010	GGTGAGAAGGGC[G/A]GGGAGAAACCCA	
		G2244T		intron2	8	18	21	47	0.362	0.638	ATTGTAACCCTA[G/T]TCATTCATTAGC	rs2070699
		T2252A		intron2	46	1	0	47	0.989	0.011	CCTAGTCATTCA[T/A]TAGCGCTGGCTC	
		T3609C		intron2	33	12	2	47	0.830	0.170	AAGACTATTAAT[T/C]ACACTAATATAG	rs1800543
		A3730G	Glu106Glu	exon3	0	1	46	47	0.011	0.989	AACAGACCGTGA[A/G]AATAGATGCCAA	rs 5369
		T5629A		intron4	47	1	0	48	0.990	0.010	GGGTGATTTTTT[T/A]AAAATAACATTT	
		G5727T	Lys198Asn	exon5	31	14	2	47	0.809	0.191	GCTGAAAGGCAA[G/T]CCCTCCAGAGAG	rs 5370

Abbreviation: SNPs, single nucleotide polymorphisms.
By Gene Cards. Version: 2.25, released 3 July, 2002.

Table 2 Subject characteristics

	All (n = 630)	*Male (n = 340)*	*Female (n = 290)*	P-*value*
Age (years)	64.6±10.6	63.3±11.3	66.0±9.6	0.0015
Height (cm)	160.0±8.7	165.8±6.4	153.1±5.5	<0.0001
Weight (kg)	62.9±11.6	68.5±10.6	56.4±9.1	<0.0001
Heart rate (b.p.m.)	64.0±10.7	62.0±9.4	66.2±11.8	<0.0001
Systolic blood pressure (mm Hg)	138.8±17.1	137.0±15.8	140.9±18.3	0.0042
Diastolic blood pressure (mm Hg)	82.7±10.3	83.2±10.2	82.1±10.5	0.1799
Mean IMT (mm)	0.83±0.16	0.83±0.16	0.84±0.17	0.4634
Plaque score	3.13±4.76	3.57±5.18	2.61±4.17	0.0131
baPWV (cm/s)	1786.2±309.1	1755.7±297.7	1822.0±318.8	0.0071
ABI	1.12±0.08	1.13±0.09	1.11±0.07	0.0018
CRP (mg/dl)	0.15±0.28	0.17±0.20	0.14±0.30	0.1728
HbA_{1c} (%)	5.63±0.80	5.66±0.77	5.58±0.83	0.2259
Total cholesterol (mg/dl)	203.0±35.2	196.7±30.4	210.4±39.0	<0.0001
Triglyceride (mg/dl)	138.3±125.3	152.4±149.7	121.5±85.3	0.0020
HDL-cholesterol (mg/dl)	52.7±15.2	48.7±13.0	57.4±16.3	<0.0001
Smoking (current/past/never)	69/211/339	59/183/89	10/28/250	<0.0001
Anti-hypertensive medication (%)	570/630 (90.5%)	308/340 (90.6%)	262/290 (90.3%)	0.9174

Abbreviations: ABI, ankle brachial index; baPWV, brachial-ankle pulse wave velocity; CRP, C-reactive protein; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; Mean IMT, mean intima-media thickness.
Values are expressed as the means±s.d. *P* ; Student's *t*-test (male vs female).

Table 3a Comparison between SNPs of ET-1 genes and baPWV in male subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominamt*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	251	1763.8±301.0	0.4250	0.6509	0.7682
			3A4A	81	1730.3±291.5			
			4A4A	5	1795.3±271.3			
	rs2070699	T/G	TT	104	1768.1±341.2	0.6029	0.3509	0.2902
			TG	158	1731.8±265.6			
			GG	76	1787.3±297.1			
	rs5370	G(Lys)/T(Asn)	GG	182	1759.6±308.5	0.8425	0.8318	0.5438
			GT	134	1758.8±286.2			
			TT	23	1720.2±284.5			
EDNRA	rs5333	T/C	TT	182	1746.8±307.0	0.5958	0.4479	0.2086
			TC	130	1752.5±269.4			
			CC	23	1830.1±369.9			
EDNRB	rs 5351	A/G	AA	107	1706.6±285.1	0.0409 (0.4499)*	0.0004 (0.0044)*	0.0001 (0.0011)*
			AG	162	1736.1±277.7			
			GG	65	1882.2±332.7			
	rs3818416	G/T	GG	305	1759.9±301.1	0.2393	0.3593	0.2593
			GT	28	1708.0±260.2			
			TT	3	1560.5±241.2			
ECE1	rs212526	C/T	CC	247	1746.9±294.9	0.4798	0.7583	0.9557
			CT	82	1775.1±298.4			
			TT	7	1747.6±415.2			
	rs212528	T/C	TT	198	1724.6±292.4	0.0311 (0.3421)*	0.0246	0.3099
			TC	122	1810.8±298.3			
			CC	16	1679.9±308.2			
	rs213045	G/T	GG	102	1732.0±282.7	0.3865	0.3293	0.3737
			GT	174	1776.5±305.3			
			TT	59	1722.0±301.3			
	rs2038089	A/G	AA	153	1773.4±300.4	0.3051	0.0821	0.0262 (0.2882)*
			AG	138	1764.3±304.3			
			GG	43	1661.1±253.9			
ECE2	rs2272471	C/T	CC	94	1778.0±303.1	0.3573	0.6116	0.9717
			CT	164	1739.8±282.3			
			TT	76	1755.1±324.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor; *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

Table 3b Comparisons between ET-1 gene SNPs and baPWV in female subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*baPWV (cm/s)*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	198	1831.0±329.4	0.4510	0.7278	0.9152
			3A4A	84	1798.0±305.9			
			4A4A	5	1836.6±199.9			
	rs2070699	T/G	TT	80	1845.0±367.1	0.4673	0.7631	0.7183
			TG	139	1816.2±305.3			
			GG	69	1810.8±289.1			
	rs5370	G (Lys)/T(Asn)	GG	147	1843.9±321.8	0.2519	0.4711	0.5298
			GT	116	1795.2±316.4			
			TT	26	1825.8±319.9			
EDNRA	rs5333	T/C	TT	153	1796.2±306.1	0.1163	0.1601	0.5298
			TC	116	1867.4±331.3			
			CC	17	1776.6±342.9			
EDNRB	rs 5351	A/G	AA	85	1859.2±315.1	0.2257	0.3921	0.3211
			AG	145	1817.9±339.8			
			GG	56	1785.8±268.3			
	rs3818416	G/T	GG	255	1822.4±325.2	0.8168	0.3676	(–)
			GT	29	1821.6±270.6			
			TT	1	2277.0			
ECE1	rs212526	C/T	CC	208	1827.0±308.5	0.7909	0.4074	0.1873
			CT	67	1835.0±360.3			
			TT	11	1698.1±257.9			
	rs212528	T/C	TT	184	1833.1±320.8	0.5150	0.4206	0.3855
			TC	86	1791.7±307.6			
			CC	16	1891.4±371.4			
	rs213045	G/T	GG	93	1834.6±369.1	0.6899	0.4138	0.2837
			GT	142	1801.0±281.4			
			TT	50	1867.8±326.9			
	rs2038089	A/G	AA	124	1821.2±322.9	0.8902	0.9691	0.8109
			AG	131	1824.3±321.9			
			GG	24	1839.2±323.7			
ECE2	rs2272471	C/T	CC	73	1795.8±343.4	0.3612	0.5926	0.4611
			CT	144	1828.5±314.6			
			TT	68	1850.3±304.4			

Abbreviations: baPWV, brachial-ankle pulse wave velocity; SNPs, single nucleotide polymorphisms.
P-value (dominant); major vs hetero+minor, *P*-value (additive); major vs heterozygote vs minor, *P*-value (recessive); minor+hetero vs major.

Screening of genetic variations in EDN1 EDNRA, EDNRB, ECE1 and ECE2

We isolated genomic DNA from the peripheral blood leukocytes of 630 subjects and directly sequenced the entire coding region of the endothelin-1 gene (*EDN1*). The results of the *EDN1* screening are shown in Table 1. Finally, we selected three SNPs in the *EDN1*. We selected SNPs of the endothelin type A receptor gene (*EDNRA* rs5333), endothelin type B receptor gene (*EDNRB* rs5351, rs3818416), endothelin converting enzyme-1 gene (*ECE1* rs212526, rs212528, rs213045, rs2038089) and endothelin converting enzyme-2 gene (*ECE2* rs2272471) from a public database (dbSNP http://www.ncbi.nlm.nih.gov/SNP/). SNPs with a minor allele frequency of greater than 5% were genotyped using the TaqMan-PCR method described previously.[16] The representative SNPs were genotyped when they were linkage disequilibrium (LD: r^2 over 0.5). The LD was calculated between each SNP. The primers and probes used in the TaqMan-PCR system are available upon request.

Statistical analysis

Values are expressed as means ±s.d. and were analyzed using a Student's *t*-test and a χ^2-test where appropriate. Hardy–Weinberg equilibrium was assessed by χ^2analysis, and we considered *P*-values less than 0.05 to be statistically significant. The levels of the *P*-values were adjusted by Bonferroni correction). The LD between each SNP was checked using Haploview version 4 (http://www.broad.mit.edu/mpg/haploview/). The association of genotypes with blood pressure, IMT and PS of carotid arteries and baPWV was examined by simple regression analysis and then investigated using a logistic regression model that adjusted for confounding factors. The distribution of plaque score (PS) was not normal, so we compared the prevalence of severe PS ($\geqslant$10.1)[17] for each allele. All statistical analyses were performed using Stat-View version 5.0 (SAS Institute Inc., Cary, NC, USA).

Results

Patient Characteristics and the Correlation between baPWV and Clinical Parameters

The characteristics of the subjects at baseline are summarized in Table 2. Significant differences were apparent between men and women in age, height, weight, heart rate (HR), systolic blood pressure (SBP), plaque score (PS), baPWV and ABI and lipid

Table 4a Comparisons between ET-1 gene SNPs and mean IMT in male subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*Mean IMT*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	250	0.824±0.160	0.7936	0.1199	0.0400 (0.4400)*
			3A4A	81	0.821±0.152			
			4A4A	5	0.970±0.148			
	rs2070699	T/G	TT	104	0.815±0.143	0.3957	0.6548	0.5235
			TG	157	0.829±0.163			
			GG	76	0.837±0.170			
	rs5370	G (Lys)/T(Asn)	GG	181	0.832±0.154	0.4286	0.5953	0.7007
			GT	134	0.815±0.162			
			TT	23	0.838±0.174			
EDNRA	rs5333	T/C	TT	181	0.821±0.160	0.4330	0.0023 (0.0253)*	0.0005 (0.0055)*
			TC	130	0.816±0.146			
			CC	23	0.937±0.179			
EDNRB	rs 5351	A/G	AA	107	0.830±0.164	0.8131	0.0104 (0.1144)*	0.0059 (0.0649)*
			AG	161	0.805±0.147			
			GG	65	0.875±0.168			
	rs3818416	G/T	GG	304	0.828±0.161	0.5352	0.7307	0.5119
			GT	28	0.814±0.130			
			TT	3	0.767±0.161			
ECE1	rs212526	C/T	CC	246	0.832±0.161	0.3202	0.5493	0.4919
			CT	82	0.786±0.157			
			TT	7	0.814±0.152			
	rs212528	T/C	TT	198	0.826±0.156	0.9406	0.9714	0.8410
			TC	121	0.828±0.165			
			CC	16	0.819±0.153			
	rs213045	G/T	GG	102	0.831±0.179	0.7299	0.6596	0.3631
			GT	174	0.829±0.154			
			TT	58	0.809±0.134			
	rs2038089	A/G	AA	152	0.830±0.161	0.7842	0.8860	0.6774
			AG	138	0.828±0.144			
			GG	43	0.816±0.195			
ECE2	rs2272471	C/T	CC	93	0.820±0.160	0.6244	0.8500	0.9127
			CT	164	0.832±0.161			
			TT	76	0.826±0.153			

Abbreviations: SNPs, single nucleotide polymorphisms; IMT, intima-media thickness.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor; *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

profiles. Almost all subjects were treated with antihypertensive agents such that the ratio of treated patients did not differ between males and females.

We analyzed the correlations between baPWV, mean IMT, max IMT, PS of carotid arteries and the clinical parameters of male and female patients. BaPWV significantly correlated with age, height, weight, SBP, DBP, mean BP, HR and HbA_{1c}. In contrast, baPWV was not associated with serum creatinine, C-reactive protein, or ABI. The mean IMT and PS of carotid arteries significantly correlated with age, height, HbA_{1c} and HDL-Chol. All indices of atherosclerosis strongly associated with age, height and BP. Among these indices, IMT and PS showed weaker association with weight and BP than baPWV.

Correlation between baPWV and SNPs in ET-1 family genes

We studied 11 SNPs in total, including three of *EDN1*, one of *EDNRA*, two of *EDNRB*, four of *ECE1* and one of *ECE2*. We found no tight LD between the 11 analyzed SNPs. We analyzed the association of baPWV with ET-1 SNPs in all subjects, both male and female. As shown in Table 3a, we detected significant differences in baPWV in comparing additive, dominant, or recessive models in *EDNRB*-rs5351 (exon 6), *ECE1*- rs212528 (intron 3) and rs2038089 (intron 17) in male subjects. Finally, only *EDNRB* -rs5351 positively associated with baPWV after performing a Bonferroni correction. No SNPs were significantly associated with baPWV in female subjects (Table 3b).

Mean IMT, max IMT, plaque score of carotid arteries and ET-1 SNPs

The results of comparing additive, dominant, or recessive models for mean IMT in each SNP are shown in Tables 4a and 4b. Only *EDNRA*-rs5333 positively associated with mean IMT after performing a Bonferroni correction, and this association was only apparent in male subjects. With regard to max-IMT, *EDNRA*-rs5333, *EDNRB* -rs5351 and *ECE1*- rs2038089 showed a positive association, but the association was not significant after Bonferroni correction (data not shown). In comparing the prevalence of severe PS ($\geqslant$10.1) for each allele, no

Table 4b Comparisons between ET-1 gene SNPs and mean IMT in female subjects

Genes	*SNPs*	*Allele1/Allele2*		n	*Mean IMT*	P-*dominant*	P-*additive*	P-*recessive*
EDN1	A201- (4A/3A)	3A/4A	3A3A	196	0.841±0.175	0.6400	0.1625	0.0706
			3A4A	83	0.828±0.152			
			4A4A	5	0.700±0.079			
	rs2070699	T/G	TT	79	0.839±0.170	0.8018	0.1429	0.0546
			TG	137	0.849±0.174			
			GG	69	0.801±0.152			
	rs5370	G (Lys)/T(Asn)	GG	144	0.835±0.168	0.9802	0.3176	0.1531
			GT	116	0.846±0.172			
			TT	26	0.835±0.168			
EDNRA	rs5333	T/C	TT	152	0.826±0.156	0.3386	0.0463 (0.5093)*	0.1527
			TC	115	0.858±0.182			
			CC	17	0.759±0.139			
EDNRB	rs 5351	A/G	AA	84	0.836±0.152	0.9740	0.9909	0.9095
			AG	144	0.834±0.171			
			GG	56	0.837±0.186			
	rs3818416	G/T	GG	253	0.830±0.167	0.1547	0.2753	(–)
			GT	29	0.872±0.178			
			TT	1	1.000			
ECE1	rs212526	C/T	CC	206	0.834±0.168	0.9034	0.6696	0.4238
			CT	67	0.844±0.169			
			TT	11	0.795±0.159			
	rs212528	T/C	TT	183	0.831±0.158	0.5586	0.5997	0.5508
			TC	86	0.849±0.182			
			CC	15	0.810±0.205			
	rs213045	G/T	GG	92	0.826±0.162	0.5215	0.7194	0.4975
			GT	141	0.836±0.162			
			TT	50	0.850±0.196			
	rs2038089	A/G	AA	126	0.818±0.166	0.1171	0.2132	0.2300
			AG	130	0.845±0.170			
			GG	24	0.875±0.174			
ECE2	rs2272471	C/T	CC	71	0.847±0.186	0.5061	0.0360 (0.3960)*	0.0327 (0.3597)*
			CT	144	0.812±0.154			
			TT	68	0.874±0.171			

Abbreviations: SNPs, single nucleotide polymorphisms; IMT, intima-media thickness.
P-value (dominant), major vs hetero+minor; *P*-value (additive), major vs heterozygote vs minor, *P*-value (recessive), minor+hetero vs major.
*Bonferroni correction (× 11).

SNPs were positive in either male or female subjects after Bonferroni correction (Table 5).

Association of SNPs in ET-1 family genes with severe atherosclerosis

We have compared the atherosclerosis parameters and the background of each genotype of *EDNRA*-rs5333, *EDNRB*-rs5351, *ECE1*-rs212528 and rs2038089. These four SNPs showed significant association with atherosclerotic indices, including baPWV, PS and IMT. They had no association with atherosclerotic risk factors, such as HbA1c, TG, HDL-chol, except *EDNRA*-rs5333 which showed association with TG.

We divided the male subjects in three ways: a rapid or slow group based on the average baPWV (rapid: ⩾1756 cm/s, slow: <1756 cm/s), averaged mean-IMT (severe: ⩾0.86 mm, mild: <0.86 mm) and a severe or mild atherosclerotic group using plaque scores (severe: ⩾10.1, mild: <10.1). We performed logistic regression analysis on the progression of baPWV, mean-IMT and PS. Multiple logistic regression analysis indicated that GG in *EDNRB* -rs5351, with higher baPWV and PS, was an independent risk factor in male subjects (Tables 6a–c).

Discussion

The human ET-1 gene was cloned and sequenced in 1989 by Inoue *et al.*[18] Recent studies have examined the relationship between polymorphisms of ET-1 and BP. Tiret *et al.*[19] indicated that a G/T polymorphism with an amino acid substitution (Lys → Asn) at codon 198 in exon 5 of ET-1 was associated with BP in overweight Europeans, and similar results were obtained in Japanese subjects.[20,21]

In this study, we evaluated the association of 11 SNPs of ET-1 family genes with atherosclerosis in hypertensive patients. We found a significant correlation between baPWV and *EDNRB*-rs5351 and between mean IMT of carotid arteries and *EDNRA*-rs5333 in male, but not female hypertensive patients after Bonferroni correction; however, EDNRA-rs5333 was not significantly associated with severe IMT thickening after multiple logistic regression analysis. Thus, *EDNRB*-rs5351 was the most suscep-

Table 5 Genotype distribution among the subjects with severe (≥10.1) or mild atherosclerosis (by plaque scores)

				Male				*Female*			
Genes	*SNPs*	*Allele (major/minor)*	*Genotype*	*Mild*	*Severe*	χ^2	P-*value*	*Mild*	*Severe*	χ^2	P-*value*
EDN1	A201- (4A/3A)	3A/4A	3A3A	223	22	0.970	0.6156	184	11	2.668	0.2635
			3A4A	71	9			80	3		
			4A4A	4	1			4	1		
	rs2070699	T/G	TT	92	10	0.275	0.8714	74	4	1.660	0.4361
			TG	143	14			131	6		
			GG	64	8			63	6		
	rs5370	G (Lys)/T(Asn)	GG	161	18	0.082	0.9600	136	7	1.733	0.4205
			GT	120	12			109	7		
			TT	19	2			23	3		
EDNRA	rs5333	T/C	TT	160	19	0.906	0.6357	145	7	1.733	0.4204
			TC	116	10			106	8		
			CC	20	3			15	2		
EDNRB	rs 5351	A/G	AA	99	4	9.898	0.0071 (0.0781)*	79	4	2.740	0.2541
			AG	144	16			137	7		
			GG	52	12			50	6		
	rs3818416	G/T	GG	269	29	0.354	0.8376	237	15	0.105	0.9487
			GT	25	3			27	2		
			TT	3	0			1	0		
ECE1	rs212526	C/T	CC	217	23	0.171	0.9179	192	13	0.744	0.6894
			CT	74	8			63	4		
			TT	6	1			11	0		
	rs212528	T/C	TT	180	16	1.354	0.5082	171	11	1.102	0.5763
			TC	103	14			80	6		
			CC	14	2			15	0		
	rs213045	G/T	GG	92	8	1.447	0.4851	88	4	1.569	0.4564
			GT	154	16			130	11		
			TT	50	8			47	2		
	rs2038089	A/G	AA	126	23	9.901	0.0071 (0.0781)*	121	4	3.383	0.1843
			AG	130	7			119	11		
			GG	39	2			23	1		
ECE2	rs2272471	C/T	CC	82	7	4.258	0.1190	66	5	1.914	0.3840
			CT	150	13			137	6		
			TT	63	12			62	6		

Abbreviation: SNPs, single nucleotide polymorphisms.
Men and women were divided into three groups for each genotype.
*Bonferroni correction (× 11).

tible endothelin-related SNP associated with atherosclerosis in male hypertensives. With regard to the gender differences between baPWV and/or arteriosclerosis and ET-1 family gene polymorphisms, one possible explanation is that the effect of ET-1 on vasoconstriction and atherosclerosis may differ between males and females. Tatchum-Talom *et al.*[22] described the vasoconstrictive effect of ET-1 as much greater in male rats than in female rats. Alternatively, oestrogen may reduce the vasoconstriction induced by ET-1.[23] Our current findings indicate that there are differences in the progression of atherosclerotic changes among hypertensive patients that depend on the genotypes of ET-1 family genes. Therefore, our findings provide important information regarding the use of hypertensive agents. Hypertensive agents should perhaps be prescribed after taking the polymorphisms in specific patients into consideration.

Lajemi *et al.*[24] showed that the *EDNRA* -231A/G and *EDNRB* 30G/A gene polymorphisms influence PWV in women, and the *EDNRB* 30G/A genotype related to the level of radial artery parameters in men. They suggested that these genes were involved in arterial stiffness. Funalot *et al.*[25] showed that *ECE 1B* C338A and *EDN1* Lys198Asn work together to modulate BP levels in women. Of the three SNPs tested in this study, only *EDNRB*-rs5351 was associated with baPWV and PS. This may be because baPWV reveals a more functional change, while PS indicates a more structural change.

In this study, most patients were treated with antihypertensive agents, some of which might affect PWV either directly or indirectly. We did not have detailed information on the drugs being taken by each subject, which could be seen as a limitation on our study. However, it has been reported that evaluations of PWV for monitoring arterial stiffness and in developing risk assessment strategies for hypertensive patients are useful.[26]

It will be important to determine serum ET-1 levels in patients to examine whether the cause of differences in baPWV or carotid arteriosclerosis between genotypes is dependent on only the

Table 6a Logistic regression analysis of baPWV and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	0.99	(0.544–1.804)	0.9747	—	—	—
(AA-GG)	2.353	(1.110–4.989)	0.0256	—	—	—
ECE1- rs212528 (TT-TC)	—	—	—	1.833	(1.058–3.176)	0.0307
(TT-CC)	—	—	—	0.541	(0.146–2.003)	0.3575
Age	1.105	(1.070–1.142)	<0.0001	1.11	(1.075–1.147)	<0.0001
Height	0.937	(0.889–0.986)	0.0132	0.936	(0.889–0.985)	0.0118
Weight	1.006	(0.975–1.038)	0.7156	1.002	(0.970–1.034)	0.9137
Mean BP	1.071	(1.042–1.101)	<0.0001	1.071	(1.042–1.100)	<0.0001
HR	1.022	(0.993–1.052)	0.1323	1.023	(0.995–1.052)	0.1032

Abbreviations: baPWV, brachial-ankle pulse wave velocity; BP, blood pressure; ET-1, endothelin-1; HR, heart rate.
The average baPWV of male patients was 1756 cm/s. Rapid group, ⩾baPWV 1756 cm/s; slow group, <1756 cm/s.

Table 6b Logistic regression analysis of plaque scores and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRB- rs5351 (AA-AG)	3.255	(0.898–11.802)	0.0725	—	—	—
(AA-GG)	5.017	(1.308–19.239)	0.0187	—	—	—
ECE1- rs2038089 (AA-AG)	—	—	—	0.334	(0.132–0.844)	0.0205
(AA-GG)	—	—	—	0.356	(0.076–1.666)	0.1895
Age	1.09	(1.032–1.152)	0.0020	1.097	(1.038–1.160)	0.0010
Height	0.984	(0.907–1.068)	0.7006	0.988	(0.912–1.071)	0.7748
Weight	0.962	(0.906–1.021)	0.2047	0.956	(0.901–1.014)	0.1333
DBP	1.031	(0.986–1.077)	0.1843	1.039	(0.994–1.086)	0.0911

Abbreviations: DBP, diastolic blood pressure; ET-1, endothelin-1.
The severe atherosclerotic group of male patients refers to PS⩾10.1. Rapid group, ⩾PS 10.1; slow group, <10.1.

Table 6c Logistic regression analysis of mean-IMT and ET-1 gene polymorphisms adjusting for clinical parameters in male patients

	Odds ratio	*95% CI*	P-*value*	*Odds ratio*	*95% CI*	P-*value*
EDNRA- rs5333 (TT-TC)	1.066	(0.655–1.737)	0.7966	—	—	—
(TT-CC)	2.328	(0.846–6.406)	0.1018	—	—	—
EDNRB- rs5351 (AA-AG)	—	—	—	0.770	(0.449–1.319)	0.3409
(AA-GG)	—	—	—	1.349	(0.686–2.653)	0.3861
Age	1.049	(1.022–1.076)	0.0003	1.052	(1.025–1.081)	0.0002
Height	1.008	(0.969–1.048)	0.6995	1.008	(0.969–1.049)	0.6853
DBP	0.989	(0.965–1.014)	0.3835	0.992	(0.967–1.017)	0.5110
HbA_{1c}	1.188	(0.872–1.619)	0.2735	1.270	(0.932–1.730)	0.1301
HDL-CHOL	0.976	(0.958–0.995)	0.0115	0.979	(0.959–0.996)	0.0150

Abbreviations: ET-1, endothelin-1; IMT, intima-media thickness.
The average mean-IMT in male patients was 0.86 mm. Severe group, ⩾mean-IMT 0.86 mm; mild group, <0.86 mm.

ET-1 level or on the interaction of several hormonal systems. The negative vascular effects of ET-1 may contribute to the pathogenesis of hypertension and its complications in black patients.[27] Unfortunately, we did not have enough data regarding serum ET-1 levels to analyze the relationship between serum levels and ET-1 family gene polymorphisms.

It is also important to examine the influence of menopause on atherosclerosis in female subjects. However, most female subjects in the present study were older than 60 years, so it was impossible to clarify the influence of menopause in this study.

Any synergetic effects of polymorphisms on baPWV and PS should also be evaluated. In male subjects, baPWV values were slower in those with TT than with TC+CC of *ECE1*-T/C-rs212528, and were also slower in those with AA than with AG+GG of *EDNRB*-A/G-rs5351. We therefore compared baPWVs of subjects with both TT of *ECE1*-rs212528 and AA of *EDNRB*-rs5351 to those of subjects with both CC of *ECE1*-rs212528 and GG of *EDNRB*-rs5351. However, we did not obtain a stronger correlation for combined gene types than single genotypes (data not shown). We obtained similar results by analyzing IMT and PS.

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What is known about this topic

- ET-1 is thought to play important roles in the development of atherosclerosis through endothelial dysfunction and proliferation of vascular smooth muscle cells.
- There have been various studies of the relationship between polymorphisms of ET-1 and BP.

What this study adds

- EDNRB-rs5351 in exon6 might contribute to the progression of atherosclerosis in male patients with EHT.
- In future, an evaluation of these polymorphisms may be valuable for the treatment of hypertensive and/or atherosclerotic patients.

Abbreviations: ET-1, endothelin-1; BP, blood pressure; EHT, essential hypertension.

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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 $115\pm28\,g/m^2$, 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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