Endothelial impairment and bone marrow-derived CD34$^+$/133$^+$ cells in diabetic patients with erectile dysfunction

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ABSTRACT

Aims/Introduction: The present study was undertaken to determine vascular endothelial impairment and endothelial progenitor cells (EPCs) in patients with type 2 diabetes mellitus and erectile dysfunction (ED).

Materials and Methods: A total of 100 type 2 diabetic men were enrolled. Flow-mediated dilatation (FMD) and anaerobic threshold (AT) were measured. Also, EPCs in the peripheral blood were determined by flow cytometry.

Results: In the 42 ED diabetic patients, FMD and AT were significantly less than those in the 58 patients with normal erectile function (FMD 2.84 vs 3.82%, $P = 0.038$, and AT 11.2 vs 12.7 mL/kg/min, $P = 0.022$). Exercise tolerance significantly increased the number of EPCs in the patients with and without ED (49–60 cells/100 μL, $P = 0.015$, and 72–99 cells/100 μL, $P = 0.003$). In the diabetic patients without autonomic neuropathy, FMD was significantly reduced in the patients with ED than those without ED ($P = 0.015$). In response to exercise tolerance, the number of EPCs increased in both the diabetic patients with ED ($P = 0.003$) and without ED ($P = 0.007$). In contrast, in the diabetic patients with autonomic neuropathy, there was no difference in FMD between the patients with and without ED. The exercise tolerance increased the number of EPCs in the patients without ED ($P = 0.023$), but it disappeared in those with ED.

Conclusions: ED diabetic patients have endothelial impairment during the early period of diabetic complications, whose deranged endothelial function is concomitantly repaired by promoting bone marrow-derived EPCs. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2012.00230.x, 2012)

KEY WORDS: Diabetes mellitus, Endothelial progenitor cells, Erectile dysfunction

INTRODUCTION

Cardiovascular disorders are major prognostic determinants for diabetic patients, because cardiovascular death occupies approximately 50% of mortality in diabetic patients. Atherosclerotic events are not always related to the duration of diabetes mellitus and progression of diabetic microangiopathic complications. Recent studies have shown that inflammatory changes in the vascular wall are involved in developing atherosclerosis. Damaged endothelial cells release cytokines and growth factors, and macrophages and adhesion molecules accumulate into subendothelial space of the injured region, promoting the atherogenic process[1]. There is a high prevalence of ischemic heart diseases in patients with erectile dysfunction (ED) as compared with those with normal erectile function[2,3]. It is not clear whether atherosclerotic disorders are profoundly accelerated in diabetic patients with ED. Thus, it is of value to evaluate the prevalence and progression of endothelial dysfunction and ED in patients with diabetes mellitus.

Bone marrow-derived endothelial progenitor cells (EPC) can have beneficial effects on angiogenesis and vascular repair[4–7]. In ischemic heart diseases, EPC increased transiently in systemic circulation[8]. Also, transplanted bone marrow-derived CD34$^+$/133$^+$ stem cells promote contraction of the left ventricle in animal models and patients with acute myocardial infarction[9,10]. Endothelial impairment is classified into an initial stage of vascular derangement. It is little known that induction of bone marrow-derived EPC occurs in such an early stage of endothelial damage. Because exercise training can increase the number of circulating EPC in angina pectoris and acute myocardial infarction[11,12], it is interesting to examine endothelial repair by EPC.

In the present study, we determined the relationship between endothelial dysfunction and ED in patients with type 2 diabetes mellitus. In addition, whether mobilization of EPC in systemic circulation occurs in diabetic patients with endothelial impairment was examined.

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MATERIALS AND METHODS

Patients

A total of 100 patients with type 2 diabetes mellitus were enrolled in the present study between May 2006 and February 2008. They were collected from the outpatient clinic of Jichi Medical University Saitama Medical Center in Saitama, Japan. They were all the male patients aged 62.8 ± 11.1 years (mean ± SD) ranging from 26 to 80 years. Type 2 diabetes mellitus was diagnosed by the Japan Diabetes Society criteria. Hemoglobin A_{1c} (HbA_{1c}; National Glycohemoglobin Standardization Program [NGSP]) was 7.8 ± 1.6%, and the duration of diabetes mellitus was 13.5 ± 8.2 years. A total of 45 patients had hypertension, 26 had dyslipdemia and 36 had obesity. A total of 48 patients were current smokers. A total of 42 patients had erectile dysfunction (ED). We excluded the following patients: (i) those receiving hemodialysis treatment, (ii) those taking maintenance medication of nitroglycerin; (iii) those with infectious diseases; (iv) those with a malignancy; and (v) those with a past history of intrapelvic system surgery. Blood samples were collected from the patients in the sitting position after an overnight fast to determine hemoglobin A_{1c} (HbA_{1c}), serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride and creatinine at the time of visiting the outpatient clinic. Bone marrow-derived $CD34^+/CD133^+$ cells in the peripheral blood were collected from the patients in the sitting position before and after a cardiopulmonary exercise stress test (CPX). Risk factors for atherosclerosis were defined as follows: hypertension was defined as systolic blood pressure of greater than 140 mmHg, diastolic blood pressure of greater than 90 mmHg, or the patient having taken antihypertentive agents. Dyslipidemia was defined as a total cholesterol level of greater than 220 mg/dL, a high-density lipoprotein cholesterol level of less than 40 mg/dL and a triglyceride level of greater than 150 mg/dL, or the patient having taken either statins or fibrates. Obesity was defined as body mass index of greater than 25. A current smoker was defined as a patient who smoked more than one cigarette per day within 3 months. The patients who had erectile dysfunction were defined by the questionnaire survey, which included symptoms of diabetic neuropathy and erectile dysfunction. We examined the international index of erectile function (IIEF-5; fifth version,) in the present patients[13]. The patients who had erectile dysfunction were defined as an IIEF-5 score of less than 21 points. According to the questionnaire, 58 patients had normal erectile function, and 42 had ED. Diabetic retinopathy was diagnosed by an ophthalmologist. Diabetic nephropathy was defined as micro- and/or macroalbuminuria, or an estimated glomerular filtration rate (eGFR) level of less than 60 mL/min. According to the progression of nephropathy, the diabetic patients were divided into stages 1–5 based on the classification of diabetic nephropathy by the Research Committee of the Japanese Ministry of Health, Labor and Welfare for Disorders of diabetes mellitus[14]. Diabetic autonomic neuropathy was determined by composite parameter of the coefficient of variation of the R-R interval (CVRR), eGFR and retinopathy. Namely, the diabetic patients who had a CVRR of less than 2%, and those who had a CVRR of greater than 2% in combination with an eGFR of less than 50 mL/min/1.73 m^2 and the presence of retinopathy, resulting in a group of diabetic autonomic neuropathy. The numbers of patients taking medication for diabetes mellitus, hypertension and dyslipidemia are summarized in Table 1. All the patients had endothelial function tests of flow-mediated dilatation (FMD) and nitroglycerin-mediated dilatation (NMD) carried out. The patients carried out the CPX, and the number of $CD34^+/133^+$ cells before and after the CPX were measured. The present study was approved by the ethical committee of Jichi Medical University for Human Studies. We obtained informed consent from the patients who joined the present protocol.

Measurements

Physical examinations included FMD, CPX and CVRR. Biochemical examinations included HbA_{1c}, serum total cholesterol, HDL cholesterol, LDL cholesterol, triglyceride, blood urea nitrogen, creatinine, adiponectin, retinol-binding protein (RBP) 4, vascular endothelial growth factor (VEGF), placental growth factor (PlGF), C-reactive protein (CRP), CD146 and high mobility group box-1 (HMGB-1). Also, the number of $CD34^+/133^+$ stem cells was determined.

Blood Samples and Assays

Blood samples were collected into tubes and centrifuged at 2000 *g* at 4°C for 15 min. The supernatants were decanted and frozen at −80°C until assayed. The value for HbA_{1c} (%) was estimated as an National Glycohemoglobin Standardization Program (NGSP) equivalent value (%) calculated by the formula HbA_{1c} (%) = HbA_{1c} (Japan Diabetes Society [JDS]) (%) + 0.4%, considering the relational expression of HbA_{1c} (JDS) (%) measured by the previous Japanese standard substance and measurement methods and HbA_{1c} (NGSP)[15]. Serum RBP4 was measured by the methods of ELISA using Human RBP4 ELISA kits (AdipoGen, Seoul, Korea). Serum adiponectin was measured using Human adiponectin ELISA kits (Otsuka Pharmaceutical Co., Tokyo, Japan). Serum VEGF was measured using human VEGF ELISA system (GE Healthcare, Buckinghamshire, UK). Serum PlGF was measured using Quantikine® Human PlGF Immunoassay (R&D Systems, Minneapolis, MN, USA). Serum HMGB-1 was measured using HMGB1 ELISA kit II (SHINO-TEST Corporation, Kanagawa, Japan). Serum CD146 was measured using CY-QUANTTM ELISA sCD146 (Biocytex, Marseille, France). Urine samples were collected in the morning at the outpatient clinic. Urinary excretion of albumin was determined by latex agglutination immunoassay (Eiken, Tokyo, Japan). Renal function was calculated as the eGFR by the Modification of Diet in Renal Disease equation (MDRD) revised for Japanese by the Japan Society of Nephrology[16]. The measurement of the number of endothelial progenitor cells (EPCs) were defined as $CD34^+/133^+$ cells by flow

cytometry. Trained technicians of Special Reference Laboratories (SRL; Tokyo, Japan), blind to the patients' information, measured the number of $CD34^+/45^+/133^+$ cells[17], using fluorescence-activated cell sorting (FACS) (FACS CaliburTM; BD Biosciences, San Jose, CA, USA) with the following antibodies: anti-CD34 FITC (Beckman Coulter Inc., Marseille, France), anti-CD45-PerCP (BD Biosciences) and anti-CD133/2 (293C3)-PE (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany). $CD34^+$ cells were analyzed using sequential gating strategies. $CD45^+$ cells were gated on a forward scatter vs side scatter to confirm mononuclear cell fraction. At least 2,000,000 events were measured in the $CD45^+$ gate. Data were analyzed using CELLQuest (BD Biosciences). After gating $CD45^+$ from whole blood, we measured the number of cells that were double-positive for CD34 and CD133.

Flow-Mediated Dilatation

Endothelial function was evaluated by flow-mediated dilatation (FMD). FMD is an arterial response to reactive hyperemia, causing endothelial-dependent dilatation[18,19]. According to the

Table 1 | Baseline clinical and laboratory findings in the diabetic patients (all male patients

	Non-ED patients (n = 58)	ED patients (n = 42)	P-value
Age (years)	61.8 ± 13.6	64.3 ± 8.0	0.26
Height (cm)	165.1 ± 6.3	166.5 ± 4.7	0.2
Weight (kg)	69.5 ± 15.1	67.3 ± 11.3	0.424
BMI	25.3 ± 4.2	24.2 ± 3.7	0.137
HbA_{1c} (%)	7.9 ± 1.9	7.7 ± 1.2	0.56
Duration of diabetes mellitus (years)	12.5 ± 7.4	14.8 ± 8.4	0.204
Systolic blood pressure (mmHg)	134.3 ± 20.7	130.0 ± 16.1	0.253
Diastolic blood pressure (mmHg)	75.4 ± 9.0	73.8 ± 10.2	0.466
Total cholesterol (mg/dL)	188.8 ± 33.6	196.2 ± 40.0	0.32
HDL cholesterol (mg/dL)	50.0 ± 13.7	48.3 ± 13.5	0.533
LDL cholesterol (mg/dL)	113.9 ± 31.1	118.2 ± 34.3	0.511
Triglyceride (mg/dL)	123.7 ± 65.2	140.8 ± 60.1	0.184
Serum creatinine (mg/dL)	0.84 ± 0.22	0.83 ± 0.18	0.718
Estimated GFR (mL/min/1.73 m^2)	77.9 ± 21.9	76.4 ± 19.1	0.723
Clinical stages of diabetic nephropathy (1/2/3A/3B/4) (n)	34/15/6/0/3	18/9/9/1/5	0.19
CVRR (%)	2.62 ± 1.77	2.63 ± 1.57	0.98
Diabetic retinopaty (±) (n)	46/12	24/18	0.017
Smoking, n (%)	25 (43.1%)	25 (59.5%)	0.198
Treatment for diabetes mellitus, n (%)			
Diet only	4 (6.9)	2 (4.8)	0.592
Sulfonylurea (gliclazide/glibenclamide/glimepride)	3/2/32 (5.2/3.4/55.2)	2/3/24 (4.8/7.1/57.1)	0.908
Biganide	23 (39.7)	16 (38.1)	0.875
Thiazolidinedione	7 (12.1)	7 (13.5)	0.513
α-Glucosidase inhibitor	12 (20.7)	14 (33.3)	0.155
Insulin	19 (32.8)	13 (31.0)	0.848
Other current medication, n (%)			
ACEI	10 (17.2)	9 (21.4)	0.598
ARB	21 (36.2)	15 (35.7)	0.96
Calcium channel blockers	13 (22.4)	14 (33.3)	0.225
Diuretics	1 (1.7)	1 (2.4)	0.817
Aspirin	8 (13.8)	8 (19.0)	0.392
Ticlopidine	2 (3.4)	3 (7.1)	0.393
Statins	20 (34.5)	10 (23.8)	0.25
Macroangiopathies, n (%)			
None	45 (77.6)	31 (73.8)	0.663
Ischemic heart disease	4 (6.9)	2 (4.8)	0.657
Cerebral vascular disease	8 (13.8)	6 (14.2)	0.944
Arteriosclerosis obliterans	1 (1.7)	3 (7.1)	0.172

Values are mean ± SD. Values are analyzed by Student's t-test or χ^2 for independence test. ACEI, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; CVRR, the coefficient of variation of the R-R interval; GFR, glomerular filtration rate; HbA_{1c}, hemoglobin A_{1c}; HDL, high-density lipoprotein; LDL, low-density lipoprotein.

procedure previously described, FMD of the right brachial artery was determined by one investigator, the first author, between 16:00 hours and 18:00 hours after 15 min rest using 15-MHz ultrasound equipment (SONOS 5500; Philips Medical Systems, Best, the Netherlands). The brachial artery in the longitudinal section just above the antecubital fossa was imaged, and internal diameters from anterior to posterior intimal interfaces were measured at the end-diastole using B-mode imaging as baseline. The diameter was determined by the mean value of three points on the ultrasound screen. Doppler measurement provided the waveforms of intravascular blood flow. Computer software installed in the ultrasound apparatus traced the waveforms automatically and calculated mean flow velocities. A pneumatic cuff was inflated on the forearm at a pressure of over 50 mmHg higher than systolic blood pressure for 5 min. The intravascular blood flow velocities and vessel diameter were measured at 20 and 60 s after cuff deflation, respectively, the same way as that at baseline. FMD was calculated as the percent increase in vessel diameter after reactive hyperemia ([maximum diameter after reactive hyperemia–baseline diameter]/baseline diameter × 100). Nitroglycerin-mediated dilatation (NMD), which is endothelium-independent dilatation, was carried out 15 min after FMD examination by using sublingual nitroglycerin 0.3-mg spray. The basal vessel diameter was measured in the same manner noted above. The maximum vessel diameter by nitroglycerin was measured 4 min after the administration, and then NMD was calculated.

Exercise Capacity

Cardiopulmonary exercise stress test was carried out between 14:00 and 15:00 hours to measure oxygen consumption at the anaerobic threshold (AT) and peak oxygen consumption (peak VO_2) using an electronically braked cycle ergometer (Ergometer 232C^R; Minato Medical Science, Osaka, Japan) at a constant rate of 60 r.p.m. The mean exercise time was approximately 8 min. During the exercise test, blood pressure and 12-lead electrocardiogram (ECG) were monitored. The work rate was increased using a 15-W/min ramp until leg fatigue, shortness of breath, significant ST depression or maximum heart rate (220 b.p.m. – age) arrival. Expired air gas was analyzed continuously using a metabolic cart (AE-300S^R; Minato Medical Science). The anaerobic threshold was determined by the V-slope method by the starting point of the non-linear increase in carbon dioxide output[20].

Heart Rate Variability (the Coefficient of Variation of the R-R Interval)

After 15 min rest in the supine position, 12-lead ECG was recorded over 100 beats at rest, and the CVRR was analyzed automatically (Cardiofax V; Nihon Kohden, Tokyo, Japan). CVRR was determined by dividing the standard deviation of all R-R intervals (SD) by the mean R-R interval (M) for the ECG recording. CVRR (%) = (SD/M) × 100.

Statistical Analysis

All values are expressed as mean ± SEM. The values were analyzed by Student's *t*-test to compare the differences between the groups. Corresponding data were analyzed by paired *t*-test. Categorical data were analyzed by χ^2 for independence test. The statistical package of Statcel Statistical Software (Second Edition; OMS Publishing, Inc., Saitama, Japan) was used for the present analysis. A *P*-value <0.05 was considered significant.

RESULTS

Table 1 shows the clinical and laboratory characteristics of the diabetic patients in the presence and absence of ED. According to the questionnaire, 58 patients had normal erectile function and 42 had ED. Except for diabetic retinopathy, there were no differences in any parameter between the two groups of patients.

Physical and biochemical examinations are shown in Table 2. FMD was 2.84 ± 0.34% in the diabetic patients with ED, a value significantly less than that of 3.82 ± 0.39% in those without ED ($P = 0.038$). However, NMD remained normal, and there was no difference in NMD between the patients with and without ED. Oxygen consumption at AT of 11.2 ± 0.4 mL/kg/min in the diabetic patients with ED was significantly lower than that of 12.7 ± 0.5 mL/kg/min in those without ED ($P = 0.022$). Basal levels of $CD34^+/133^+$ cells were comparable

Table 2 | Physical and biochemical studies in the diabetic patients

	Non-ED patients	ED patients	*P*-value
FMD (%)	3.82 ± 0.39	2.84 ± 0.34	0.038
NMD (%)	13.8 ± 0.79	12.2 ± 0.83	0.175
n	58	42	
CPX			
VO_2 at AT (mL/kg/min)	12.7 ± 0.5	11.2 ± 0.4	0.022
Peak VO_2 (mL/kg/min)	21.4 ± 0.8	19.4 ± 0.9	0.055
n	27	33	
EPCs (cells/100 mL)			
Before CPX	72 ± 12	49 ± 6	0.119
After CPX	99 ± 18**	60 ± 8*	
n	23	22	
Serum RBP4 (μg/mL)	53.2 ± 4.1	63.0 ± 6.1	0.178
Serum Adiponectin (μg/mL)	9.8 ± 1.5	8.8 ± 1.0	0.595
Serum VEGF (pg/mL)	245 ± 21	275 ± 32	0.422
Serum PlGF (pg/mL)	10.2 ± 0.7	10.7 ± 0.5	0.608
Serum hs-CRP (mg/L)	1.37 ± 0.35	2.25 ± 0.78	0.279
Serum HMGB1 (pg/mL)	3.8 ± 0.1	3.7 ± 0.2	0.791
Serum CD146 (ng/mL)	585 ± 23	553 ± 23	0.331
n	38	35	

Values are mean ± SEM. Values are analyzed by Student's *t*-test or paired *t*-test. *$P < 0.05$, **$P < 0.001$ vs the value before cardiopulmonary exercise stress test (CPX). AT, anaerobic threshold; EPCs, endothelial progenitor cells; FMD, flow-mediated dilatation; HMGB1, high mobility group box 1; hs-CRP, high sensitive C-reactive protein; NMD, nitroglycerin-mediated dilatation; PlGF, placental growth factor; RBP4, retinol-binding protein 4; VEGF, vascular endothelial growth factor; VO_2, oxygen consumption.

in both the diabetic patients with and without ED, but the exercise load significantly augmented $CD34^+/133^+$ cells in both groups ($P = 0.0003$ and $P = 0.015$). Biochemical studies showed that there were no differences in all the variables, including serum RBP4, adiponectin, VEGF, PlGF, CRP, HMGB1 and CD146 between the two groups of patients.

We divided the diabetic patients according to the development of autonomic neuropathy. Physical and biochemical studies are shown in Table 3 and Figure 1. In the diabetic patients without autonomic neuropathy, FMD was significantly reduced in the diabetic patients with ED than those without ED ($2.43 \pm 0.38\%$ vs $3.92 \pm 0.41\%$, $P = 0.015$). There was not any difference in oxygen consumption at AT and peak VO_2 between the diabetic patients with and without ED in the exercise tolerance study. In response to exercise load, the numbers of $CD34^+/133^+$ cells significantly increased from 56 ± 10 to 72 ± 15 cells/100 μL in the diabetic patients with ED ($P = 0.012$) and from 92 ± 21 to 125 ± 29 cells/100 μL in those without ED ($P = 0.007$; Figure 1). There was not any difference in serum RBP4, adiponectin, VEGF, PlGF, CRP, HMGB1 and CD146 between the presence and absence of ED in the diabetic patients. In contrast, in the diabetic patients with autonomic neuropathy, there was no difference in FMD and NMD between the diabetic patients with and without ED. The exercise tolerance study showed that oxygen consumption at AT and peak VO_2 was significantly less in the diabetic patients with ED than those without ED ($P = 0.026$ and $P = 0.036$, respectively). In both the patients with and without ED, the numbers of $CD34^+/133^+$ cells before and after the exercise load seemed likely to be comparable levels. The exercise tolerance significantly increased the numbers of $CD34^+/133^+$ cells in the patients without ED ($P = 0.023$), but it disappeared in those with ED. Otherwise, there was not any difference in serum RBP4, adiponectin, VEGF, PlGF, CRP, HMGB1 and CD146 between the diabetic patients in the presence and absence of ED.

Similarly, Table 4 shows physical and biochemical studies in association with the progression of diabetic nephropathy. In the patients whose eGFR was greater than 60 mL/min/1.73 m^2, the ED patients had lower FMD than the non-ED patients ($2.82 \pm 0.39\%$ vs $3.94 \pm 0.37\%$, $P = 0.044$). After exercise tolerance, the numbers of $CD34^+/133^+$ cells significantly increased in both the patients with ED (51 ± 7 to 63 ± 10 cells/100 μL, $P = 0.003$) and without ED (84 ± 14 to 117 ± 20 cells/100 μL, $P = 0.0003$). In contrast, in the patients who had eGFR less than 60 mL/min/1.73 m^2, there was no difference in FMD and oxygen consumption at AT and peak VO_2 between the presence and absence of ED. The exercise load also did not increase the numbers of $CD34^+/133^+$ cells in the patients with and without ED. However, a limitation of the study was the small number of patients with nephropathy in relation to the analysis of

Table 3 | Physical and biochemical studies in diabetic patients according to the development of autonomic neuropathy

	No autonomic neuropathy			Autonomic neuropathy†		
	Non-ED patients	ED patients	*P*-value	Non-ED patients	ED patients	*P*-value
FMD (%)	3.92 ± 0.41	2.43 ± 0.38	0.015	3.65 ± 0.50	2.26 ± 0.50	0.065
NMD (%)	13.1 ± 1.1	11.2 ± 1.2	0.251	13.7 ± 1.2	13.3 ± 1.3	0.816
n	29	19		24	16	
CPX						
VO_2 at AT (mL/kg/min)	12.2 ± 0.6	11.5 ± 0.5	0.385	13.1 ± 0.9	10.7 ± 0.6	0.026
Peak VO_2 (mL/kg/min)	20.2 ± 0.8	20.2 ± 0.9	0.922	22.6 ± 1.7	18.3 ± 1.5	0.036
n	16	16		10	16	
EPCs (cells/100 μl)						
before-CPX	92 ± 21	56 ± 10	0.151	45 ± 9	47 ± 7	0.872
after-CPX	125 ± 29†	72 ± 15†		60 ± 12*	55 ± 9	
n	12	11		10	12	
Serum RBP4 (μg/mL)	52.7 ± 4.5	61.5 ± 7.7	0.337	53.6 ± 6.8	62.1 ± 8.8	0.446
Serum Adiponectin (μg/mL)	7.7 ± 1.1	7.3 ± 0.7	0.721	11.3 ± 2.5	10.8 ± 1.7	0.877
Serum VEGF (pg/mL)	216 ± 27	250 ± 43	0.497	264 ± 29	298 ± 45	0.513
Serum PlGF (pg/mL)	11.0 ± 1.1	10.8 ± 0.7	0.827	9.5 ± 0.8	10.2 ± 0.7	0.515
Serum hs-CRP (mg/L)	1.13 ± 0.18	1.09 ± 0.18	0.872	1.61 ± 0.69	3.65 ± 1.66	0.218
Serum HMGB1 (pg/mL)	3.7 ± 0.2	3.6 ± 0.3	0.6	3.9 ± 0.2	3.9 ± 0.4	0.959
Serum CD146 (ng/mL)	569 ± 25	545 ± 31	0.553	603 ± 35	572 ± 31	0.528
n	17	17		19	16	

Values are mean ± SEM. Values are analyzed by Student's *t*-test or paired *t*-test. *$P < 0.05$ vs the value before cardio-pulmonary exercise stress test (CPX). †Diabetic autonomic neuropathy was determined by composite parameters of coefficient of variation of the R-R interval (CVRR), estimated glomerular filtration rate and the presence of retinopathy. AT, anaerobic threshold; EPC, endothelial progenitor cells; FMD, flow-mediated dilatation; HMGB1, high mobility group box 1; hs-CRP, high sensitive C-reactive protein; NMD, nitroglycerin-mediated dilatation; PlGF, placental growth factor; RBP4, retinol-binding protein 4; VEGF, vascular endothelial growth factor; VO_2, oxygen consumption.

Table 4 | Physical and biochemical studies in diabetic patients in association with the progression of diabetic nephropathy

	eGFR ≥ 60 mL/min/1.73 m^2			eGFR < 60 mL/min/1.73 m^2		
	Non-ED patients	ED patients	*P*-value	Non-ED patients	ED patients	*P*-value
FMD (%)	3.94 ± 0.37	2.82 ± 0.39	0.044	3.49 ± 0.55	2.82 ± 0.56	0.458
NMD (%)	14.2 ± 0.9	12.6 ± 1.0	0.235	12.7 ± 1.4	10.8 ± 2.0	0.431
n	42	33		16	9	
CPX						
VO_2 at AT (mL/kg/min)	12.8 ± 0.5	11.5 ± 0.5	0.065	12.7 ± 1.5	10.2 ± 0.6	0.106
Peak VO_2 (mL/kg/min)	21.6 ± 1.0	19.4 ± 1.1	0.159	20.7 ± 1.8	19.2 ± 1.3	0.517
n	20	22		6	9	
EPCs (cells/100 μL)						
Before CPX	84 ± 14	51 ± 7	0.049	28 ± 11	43 ± 6	0.292
After CPX	117 ± 20**	63 ± 10*		33 ± 10	46 ± 10	
n	18	18		5	4	
Serum RBP4 (μg/mL)	46.5 ± 3.6	51.5 ± 4.6	0.399	64.8 ± 8.7	93.1 ± 14.0	0.085
Serum Adiponectin (μg/mL)	8.3 ± 1.3	8.1 ± 1.0	0.893	12.3 ± 3.5	10.5 ± 2.4	0.714
Serum VEGF (pg/mL)	255 ± 22	264 ± 33	0.829	222 ± 48	308 ± 80	0.343
Serum PlGF (pg/mL)	10.8 ± 1.0	10.2 ± 0.5	0.635	9.1 ± 0.6	11.9 ± 0.8	0.009
Serum hs-CRP (mg/L)	1.54 ± 0.52	2.17 ± 1.00	0.571	1.05 ± 0.23	2.47 ± 0.98	0.097
Serum HMGB1 (pg/mL)	3.8 ± 0.2	3.9 ± 0.3	0.797	3.8 ± 0.2	3.3 ± 0.3	0.151
Serum CD146 (ng/mL)	568 ± 31	523 ± 27	0.282	615 ± 31	633 ± 29	0.699
n	26	26		12	9	

Values are mean ± SEM. Values are analyzed by Student's *t*-test or paired *t*-test. *$P < 0.05$, **$P < 0.001$ vs the value before cardiopulmonary exercise stress test (CPX). AT, anaerobic threshold; CPX, cardio-pulmonary exercise stress test; eGFR, estimated glomerular filtration rate; EPC, endothelial progenitor cells; FMD, flow-mediated dilatation; hs-CRP, high sensitive C-reactive protein; HMGB1, high mobility group box 1; NMD, nitroglycerin-mediated dilatation; PlGF, placental growth factor; RBP4, retinol-binding protein 4; VEGF, vascular endothelial growth factor; VO_2, oxygen consumption.

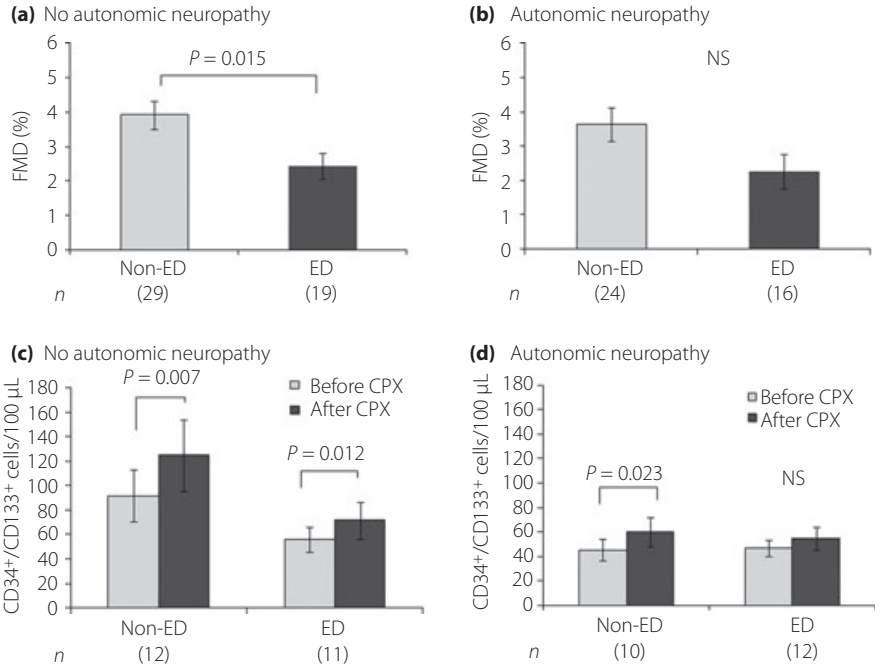

Figure 1 | (a,b) Flow-mediated dilatation (FMD) in the right brachial artery in diabetic patients with and without erectile dysfunction (ED). The patients were subgrouped into two groups by progression of autonomic neuropathy. (c,d) Alterations in bone marrow-derived endothelial progenitor cells before and after the cardiopulmonary exercise stress test in diabetic patients with and without ED. Values are means ± SEM. The numbers of patients are shown in brackets.

$CD34^+/133^+$ cells. Also, there were no differences in serum adiponectin, RBP4, VEGF, CRP, HMGB-1 and CD146 between the two groups with and without ED.

DISCUSSION

The present study showed the decreases in FMD and oxygen consumption at AT in type 2 diabetic patients with ED as compared with those who had normal erectile function. In particular, vascular endothelial dysfunction was obvious in the diabetic patients with ED whose diabetic autonomic neuropathy and nephropathy were minimal. There is a close association of endothelial dysfunction with ED in diabetic patients with minimal chronic complications. We further analyzed the past history of cardiovascular diseases in these patients, and did not find any high prevalence of ischemic heart disease and cerebral infarction in the ED diabetic patients, even if the number of patients was small. However, recent studies have reported the relationship of ED with cardiovascular diseases. Chiurlia *et al.*[2] showed that coronary atherosclerosis is severe in patients with ED. A mass study showed that ED was associated with a hazard ratio of 1.25 for subsequent cardiovascular events during the 5-year follow up in 2420 patients with ED[3]. We collected the diabetic patients with ED in the present study, and showed endothelial impairment preceded atherosclerotic disorders in the ED diabetic patients with modest chronic complications. As aforementioned, 65% of the ED patients had 5–7 points in the IIEF-5 score, thus resulting in advanced impairment of erectile function. There was no correlation between IIEF-5 score and FMD (data not shown). These findings might implicate that endothelial dysfunction links to future development of atherosclerotic disorders in the diabetic patients with ED. There was no difference in average ages between the ED and non-ED diabetic patients. We suspected that several factors, including severity of vascular endothelial dysfunction and autonomic neuropathy and others, as well as aging, could be involved in diabetic patients developing ED.

We further analyzed EPC characterized by $CD34^+/133^+$ mononuclear cells in the systemic circulation of patients with diabetes mellitus. Bone marrow-derived EPC can have beneficial effects on vascular repair and angiogenesis, and inhibit atherosclerotic process[4–7,9,10,21,22]. In the basal condition, there was no difference in the numbers of $CD34^+/133^+$ cells between the diabetic patients in the presence and absence of ED. After exercise tolerance, the numbers of $CD34^+/133^+$ cells significantly increased in the ED diabetic patients having no autonomic neuropathy or having eGFR more than 60 mL/min, and the study might suggest that bone marrow-derived stem cells protect from further development of endothelial impairment. However, bone marrow-derived stem cells no longer altered endothelial dysfunction in the ED diabetic patients who had advanced microangiopathy. Several reports showed that physical exercise induces mobilization of EPCs from bone marrow[7,11,12,23]. The mechanism for exercise-promoted EPCs is not evident, but an undetermined factor might be derived during the exercise load, and promote bone marrow to derive stem cells (EPCs). When the patients have advanced microangiopathy, this activation might disappear. Taken together, the present study might indicate that endothelial dysfunction could occur with manifestation of ED, and that also endothelial repair concomitantly proceeds by promoting bone marrow-derived EPC during the relatively early period of diabetic complications.

We measured several adipokines, growth factors and inflammatory cytokines in patients with diabetes mellitus. There was no alteration in the synthesis of endothelial growth factors under chronically developed impairment of the endothelium. No change in plasma VEGF and PlGF levels was dissociated from the findings of diminished dilating capability of the endothelium and the induction of EPC. As noted earlier, in this situation, bone marrow-derived EPC promptly promoted to repair the endothelium itself and improve endothelial function in the ED diabetic patients as well as in the non-ED diabetic patients. Endothelial derangement had a different progression among the physiological and biochemical properties in the diabetic patients. Alteration in serum RBP4 and adiponectin, which are derived from adipose tissues and partly the liver, are closely associated with diabetes mellitus and atherosclerotic disorders[24–29]. In the present study, there was no change in serum RBP4 and adiponectin levels in the ED and non-ED diabetic patients who had either autonomic neuropathy or not. The present study noted that the ED patients had no increment in atherosclerotic diseases. Endothelial impairment should reside in the preclinical state of atherosclerosis in ED diabetic patients, and might not affect serum levels of adipokines.

In conclusion, the present study showed reduced FMD and anaerobic threshold after exercise in ED diabetic patients who had no or modest autonomic neuropathy and/or nephropathy. Also, the exercise-induced increase in EPC was found in ED diabetic patients, a finding similar to those without ED. In contrast, in advanced diabetic microangiopathy, there was a reduction in FMD and exercise-induced EPC in the ED diabetic patients. These findings might indicate that ED diabetic patients have vascular endothelial impairment during the early period of diabetic complications, who concomitantly repair endothelial function by promoting bone marrow-derived EPC. Furthermore, endothelial derangement has different progression between the physiological and biochemical properties in diabetic patients.

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