Original Article

The Relationship Between Plasma Osteoprotegerin and Endothelium-Dependent Arterial Dilation in Type 2 Diabetes

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Osteoprotegerin is a recently identified inhibitor of bone resorption. Recent studies indicate that osteoprotegerin also acts as an important regulatory molecule in the vasculature. The purpose of this study was to investigate the relationship between plasma osteoprotegerin levels and endothelium-dependent arterial dilation in type 2 diabetic patients. The study subjects included 40 newly diagnosed type 2 diabetic patients and 46 healthy subjects. All patients were given insulin therapy for 6 months. Plasma osteoprotegerin concentration was measured in duplicate by a sandwich enzyme-linked immunosorbent assay method, and high-resolution ultrasound was used to measure brachial artery diameter at rest, after reactive hyperemia, and after sublingual glyceryltrinitrate. The plasma osteoprotegerin level in patients before treatment was 3.36 ± 0.32 ng/l, which was significantly higher than that in control subjects (2.38 ± 0.25 ng/l, $P < 0.001$). After 6 months of treatment, osteoprotegerin levels decreased markedly (2.83 ± 0.34 ng/l, $P < 0.001$). Flow-mediated endothelium-dependent arterial dilation in patients before treatment was 3.21 ± 0.52%, which was significantly lower than that in control subjects (4.46 ± 0.56%, $P < 0.01$), and it improved markedly after 6 months of treatment (4.03 ± 0.49%, $P < 0.01$). In multivariate analysis, osteoprotegerin was significantly associated with endothelium-dependent arterial dilation, fasting blood glucose (FBG), HbA_{1c} (A1C), and ultrasensitive C-reactive protein (CRP) at baseline ($P < 0.01$). The absolute changes in osteoprotegerin showed significant correlation with changes in endothelium-dependent arterial dilation, FBG, A1C, and CRP in diabetic patients during the course of treatment ($P < 0.01$). This study shows that plasma osteoprotegerin levels are elevated in newly diagnosed diabetic patients and are significantly associated with endothelial function. *Diabetes* **55:2126–2131, 2006**

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apo, apolipoprotein; CRP, C-reactive protein; FBG, fasting blood glucose; Lp(a), lipoprotein(a); RANKL, receptor activator of nuclear factor-κB ligand; UAER, urinary albumin excretion rate.

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Osteoprotegerin, a recently identified glycoprotein belonging to the tumor necrosis factor receptor superfamily, was originally discovered as an inhibitor of bone resorption. This inhibition is mediated through osteoprotegerin's binding and neutralization of the receptor activator of nuclear factor-κB ligand (RANKL), a strong inducer of osteoclast differentiation (1). Recently, some studies have indicated that osteoprotegerin also acts as an important regulatory molecule in the vasculature (2–6), and increased plasma osteoprotegerin concentrations are associated with coronary artery disease in nondiabetic patients (6,7). In some more recent studies in diabetic subjects, a strong association between plasma levels of osteoprotegerin and micro- and macroangiopathy was observed (8,9). Moreover, in a large observational study, plasma concentrations of osteoprotegerin were higher in diabetic than in nondiabetic subjects (10). Endothelial dysfunction is an early physiological event in atherosclerosis (11). However, to date, no data are available on the relationship between osteoprotegerin and endothelial dysfunction in diabetes. Therefore, we hypothesized that plasma osteoprotegerin level is associated with endothelial dysfunction. The purpose of this study was to investigate the relationship between plasma osteoprotegerin levels and endothelium-dependent arterial dilation in type 2 diabetic patients.

RESEARCH DESIGN AND METHODS

From January 2002 to November 2003, a total of 40 Chinese Han type 2 diabetic patients (19 men and 21 women) were selected. They had been referred to our hospital and were aged 44–60 years (mean 50 ± 6). Patients with hypertension and those with micro- and macroangiopathy, including nephropathy (urinary albumin excretion rate [UAER] >20 μg/min), retinopathy (at least one microaneurysm or hemorrhage or exudates in either eye), neuropathy (pain in extremities, paresthesias, and absent tendon reflexes and/or absent vibration sense), coronary artery disease (myocardial infarction, ischemia electrocardiogram changes, and angina), cerebrovascular disease (transient ischemic attack or stroke), and peripheral vascular disease (the abolition of one or more peripheral arterial pulse and/or intermittent claudication and/or a past history of revascularization of the lower limbs) were excluded from the study. During the same period, 46 healthy subjects (all from medical staff in our hospital) were selected as control subjects. Each subject was asked details of smoking history and family history of premature vascular disease. Cigarette smokers were defined as subjects who had smoked at least one cigarette daily for 1 year. Family history was considered positive if a first-degree relative had had clinical evidence of coronary artery disease (angina, myocardial infarction, or bypass surgery) at age ≤55 years. Subjects who were obese (BMI >30 kg/m^2) and those with malignant neoplasms, renal or liver diseases, or endocrinological disease other than diabetes were excluded from the study. Also, no patient was taking any drugs, such as estrogen supplements, thyroxine, diuretics, hypolipidemic drugs, or β-block-

ers. All subjects enrolled in the study gave informed consent. The study protocol was in agreement with the guidelines of the ethics committee at our hospital. All patients were newly diagnosed.

After diabetic dietary and regular aerobic exercise training advice, vascular and laboratory examinations were performed 1 day before and 6 months after the initiation of insulin therapy in diabetic patients. All decisions on individual insulin treatment (e.g., morning and afternoon insulin in 33 of 40 subjects; morning, afternoon, and bedtime insulin in 7 of 40 subjects; type of insulin used; and dosage adjustments to reach the treatment goal of HbA_{1c} [A1C] <7%) were left to the treating physician in the diabetes outpatient department. The control group was only studied one time.

Biochemical measurements. Venous blood samples were drawn after a 12- to 14-h overnight fast. The plasma concentrations of osteoprotegerin was measured in EDTA-plasma samples by a commercially available kit (R&D Systems, Minneapolis, MN). This assay is a sandwich enzyme-linked immunosorbent assay, using a mouse anti-human osteoprotegerin as capture antibody and a biotinylated goat anti-human osteoprotegerin for detection. Recombinant human osteoprotegerin was used for calibration, and the range of the assay was 62.5–4,000 pg/ml. Plasma samples were diluted 1:3 and measured in duplicate, and the results were averaged. The intra-assay coefficient of variation was 2–3.5%.

Measurement of serum lipids, lipoproteins, and other parameters. Serum total cholesterol, LDL cholesterol, triglycerides, and HDL cholesterol were measured enzymatically. Apolipoprotein (apo)A1 and apoB were measured by immunoturbidimetry. Serum lipoprotein(a) [Lp(a)] concentration was measured by an enzyme-linked immunosorbent assay method. Fasting blood glucose (FBG) was measured by a glucose oxidase procedure. A1C was measured by high-performance chromatography. Ultrasensitive C-reactive protein (CRP) was measured by particle enhanced immunoturbidimetric assay. UAER was measured by radioimmunoassay. Coefficients of variation for these assays were 1–2% (glucose, total cholesterol, HDL cholesterol, and A1C), 2–3% (triglycerides, LDL cholesterol, and CRP), 2–4% ($apoA_1$, apoB, and UAER), and 4–7% [Lp(a)].

Ultrasound study of the brachial artery. The vascular studies of the brachial artery were performed noninvasively, as described by us previously (12,13). High-resolution ultrasound (128XP/10 with a 7.0-MHz linear array transducer; Acuson, Mountain View, CA) was used to measure changes in arterial diameter in response to both reactive hyperemia (with increased flow producing an endothelium-dependent stimulus to vasodilation) and glyceryltrinitrate (an endothelium-independent vasodilator). The intra- and interobserver variability in our laboratory for repeated measurements of artery diameter are 0.09 ± 0.10 and 0.08 ± 0.13 mm, respectively.

The subjects rested in the supine position for 10 min before the first scan and remained supine throughout the study. The target artery (the brachial artery 2–15 cm above the elbow) was scanned in longitudinal sections, and the center of the vessel was identified when the clearest images of the anterior and posterior walls of the artery were obtained. The transmit zone was set to the level of the anterior vessel wall. Depth and gain settings were optimized to identify the lumen–to–vessel wall interface. Images were magnified with the resolution box function, leading to a television line width of ~0.05 mm. Machine settings were kept constant during each study.

Flow increase was induced by inflation of a blood pressure tourniquet placed around the forearm distal to the target artery, to 300 mmHg. The cuff was released after 5 min, and after cuff deflation the artery was scanned continuously for 90 s. We allowed 15 min for vessel recovery and then administered sublingual glyceryltrinitrate (400-μg spray), and we performed the last scan 4–5 min later. The electrocardiogram was monitored continuously.

Vessel diameter was measured by two observers unaware of clinical details and the stage of the experiment. The arterial diameter was measured at a fixed distance from an anatomical maker, such as a bifurcation, with ultrasonic calipers. Measurements were taken from the anterior to the posterior "m" line at end diastole, incident with the R-wave on the electrocardiogram. The mean diameter was calculated from four cardiac cycles. For the hyperemia scan, vessel diameter was measured 45–60 s after cuff release. Diameter changes were derived as the percent change relative to the first baseline scan (100%). Baseline blood flow (measured during the first baseline scan) was estimated by multiplying angle-corrected pulsed Doppler recordings of the flow-velocity integral by π and the square of the radius of the artery.

Statistical methods. Data are reported as the means ± SD. The difference in each parameter between before and after treatment was compared, using Student's t test (two tailed) for paired data and Student's unpaired t test for comparisons between patients and control subjects. At baseline, univariate analysis of the effects of each potential risk factor on osteoprotegerin was performed with linear regression for continuous variables [total cholesterol, triglycerides, LDL cholesterol, HDL cholesterol, triglycerides, Lp(a), apoA1, apoB, mean blood pressure, age, endothelium-dependent arterial dilation, endothelium-independent arterial dilation, vessel size, blood flow, BMI, FBG, 2-h blood glucose, A1C, and UAER] and with one-way ANOVA for categorical variables (family history, smoking, and sex). Independent association between osteoprotegerin and other independent variables was assessed by multiple regression analysis. Correlations between changes in osteoprotegerin and changes in clinical and biochemical characteristics (results of brachial artery studies in type 2 diabetes during treatment) were determined by Spearman's analysis. Lp(a) and UAER concentrations were log transformed before analysis and are reported as the median value and range. All analyses were carried out by using the statistical package SPSS10.0.

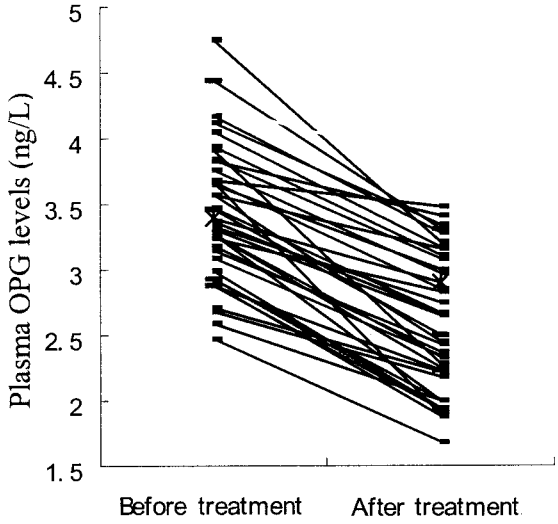

FIG. 1. Changes of osteoprotegerin (OPG) levels before and after treatment in diabetic patients.

RESULTS

The clinical characteristics and biochemical results of the control subjects and diabetic patients before and after insulin therapy are given in Table 1. Compared with control subjects, the FBG, 2-h blood glucose, A1C, total cholesterol, triglycerides, and LDL cholesterol levels in diabetic patients before treatment were significantly higher ($P < 0.05$) and decreased significantly after 6 months of treatment ($P < 0.05$). The HDL cholesterol level in diabetic patients before treatment was significantly lower than that in control subjects ($P < 0.05$), and after treatment HDL cholesterol increased slightly ($P > 0.05$).

The plasma osteoprotegerin level in patients before treatment was 3.44 ± 0.52 ng/l, which was significantly higher than that in control subjects (2.38 ± 0.25 ng/l, $P < 0.001$). After 6 months of treatment, osteoprotegerin levels decreased markedly (2.61 ± 0.55 ng/l, $P < 0.001$), which was still higher than that in control subjects ($P < 0.05$) (Table 1). As shown in Fig. 1, osteoprotegerin changes of all patients showed a marked decrease during the course of treatment.

Flow-mediated endothelium-dependent arterial dilation in patients before treatment was 3.19 ± 0.52%, which was significantly lower than that in control subjects (4.46 ± 0.56%, $P < 0.01$), and improved markedly after 6 months of treatment (4.18 ± 0.48%, $P < 0.01$), which was still lower than that in control subjects ($P < 0.05$) (Table 2). As shown in Fig. 2, endothelium-dependent arterial dilation of all patients showed a marked increase during the course of treatment. Other parameters, such as BMI, systolic blood pressure, diastolic blood pressure, and baseline vessel and glyceryltrinitrate-induced dilation, did not differ among different groups (Tables 1 and 2).

Univariate analysis showed a correlation between osteoprotegerin and endothelium-dependent arterial dilation

TABLE 1
Clinical and biochemical characteristics in control subjects and in patients with type 2 diabetes before and after insulin treatment

Variables	Control group	Diabetes before treatment	Diabetes after treatment
n	46	40	40
Age (years)	49.5 ± 5	50 ± 6	50.5 ± 6
Sex (M/F)	24/22	19/21	19/21
BMI (kg/m^2)	24.4 ± 2.5	25.2 ± 2.2	24.8 ± 3.0
Systolic blood pressure (mmHg)	122.3 ± 8.7	126.8 ± 7.6	121.4 ± 8.2
Diastolic blood pressure (mmHg)	72.9 ± 6.2	74.3 ± 7.1	75.5 ± 6.9
Positive family history	0 (0)	3 (7.5)	3 (7.5)
Smokers	6 (13.0)	5 (12.5)	5 (12.5)
FBG (mmol/l)	4.72 ± 0.46	9.87 ± 2.03*	5.81 ± 0.76†‡
2-h blood glucose (mmol/l)	6.05 ± 0.61	15.64 ± 2.96*	7.13 ± 1.27*†
A1C (%)	5.23 ± 0.38	9.18 ± 1.25*	6.33 ± 0.46†‡
Total cholesterol (mmol/l)	4.46 ± 0.33	5.80 ± 0.69*	5.12 ± 0.41§
Triglycerides (mmol/l)	1.35 ± 0.62	2.36 ± 0.84*	1.77 ± 0.74§
LDL cholesterol (mmol/l)	2.04 ± 0.36	3.53 ± 0.71*	2.76 ± 0.52§
HDL cholesterol (mmol/l)	1.28 ± 0.23	1.02 ± 0.21‡	1.11 ± 0.21
ApoA1 (g/l)	1.13 ± 0.19	1.20 ± 0.22	1.17 ± 0.24
ApoB (g/l)	0.98 ± 0.26	1.09 ± 0.19	1.17 ± 0.23
Lp(a) (mg/l)	216 (56–512)	241 (84–457)	228 (62–606)
CRP (mg/l)	1.13 ± 0.46	2.78 ± 0.77*	1.78 ± 0.68†‡
UAER (μg/min)	8.3 (1–16)	10.5 (3.3–16.8)	9.7 (5–15.6)
Osteoprotegerin (ng/l)	2.38 ± 0.25	3.44 ± 0.52*	2.61 ± 0.55†‡

Data are means ± SE, *n* (%), and median (range). *$P < 0.01$, compared with control group; †$P < 0.01$, compared with diabetes before treatment; ‡$P < 0.05$, compared with control group; §$P < 0.05$, compared with diabetes before treatment.

($r = -0.37$, $P < 0.001$), LDL cholesterol (0.22, 0.033), triglycerides (0.20, 0.041), Lp(a) (0.19, 0.046), FBG (0.29, <0.001), 2-h blood glucose (0.24, 0.01), CRP (0.34, <0.001), BMI (0.21, 0.039), and mean blood pressure (0.23, 0.01) in diabetic patients. Table 3 shows the results of multiple regression analysis of the basal levels of various clinical variables to evaluate their association with the basal level of plasma osteoprotegerin in diabetic patients. In model 1, which included endothelium-dependent arterial dilation, CRP, FBG, 2-h blood glucose, and A1C as independent variables, only endothelium-dependent arterial dilation, CRP, FBG, and A1C were found to be significant factors that were associated with plasma osteoprotegerin. When A1C was replaced by LDL cholesterol, triglycerides, Lp(a), BMI, and mean blood pressure, these variables failed to emerge as significant independent determinants of osteoprotegerin (models 2–6). Also, Spearman's analysis showed that the absolute changes in osteoprotegerin have significant correlation with the changes in endothelium-dependent arterial dilation ($P < 0.001$) (Fig. 3), FBG ($P = 0.006$) (Fig. 4), A1C ($P = 0.001$) (Fig. 5), and CRP ($P < 0.001$) (Fig. 6), and there was no significant correlation with other parameters in diabetic patients during the course of treatment.

DISCUSSION

The current study demonstrates that plasma osteoprotegerin levels are correlated with endothelium-dependent arterial dilation in type 2 diabetes. As far as we know, this is the first report on a relationship between plasma osteoprotegerin and endothelial function in diabetic patients.

The clinical coincidence of osteoporosis and vascular disease has long indicated that common mediators may adversely affect both bone metabolism and vascular integrity (14). Osteoprotegerin binds to RANKL and tumor necrosis factor–related apoptosis-inducing ligand (15), thereby inhibiting the ligation of these mediators to their receptors and inhibiting their subsequent activation of specific proinflammatory and proapoptotic signaling pathways (16). Osteoprotegerin knockout mice displayed osteoporosis and arterial calcification of the media of the aorta and renal arteries (3), and the vascular phenotype of these mice was rescued by osteoprotegerin transgene activation (17). Therefore, osteoprotegerin is considered a vascular system protective factor that prevents vascular calcification. Vascular protective effects of osteoprotegerin were also found in a study reporting that the parenteral administration of osteoprotegerin prevented

TABLE 2
The results of brachial artery studies in control subjects and in patients with diabetes before and after treatment

Variables	Control group	Diabetes before treatment	Diabetes after treatment
n	46	40	40
Baseline vessel (mm)	3.81 ± 0.53	3.78 ± 0.62	3.79 ± 0.57
Baseline flow (ml/min)	81.15 ± 32.53	80.64 ± 31.17	81.55 ± 30.26
Flow-mediated dilation (%)	4.46 ± 0.56	3.19 ± 0.52*	4.18 ± 0.48†‡
Glyceryltrinitrate-induced dilation (%)	21.65 ± 2.40	20.89 ± 2.25	21.18 ± 2.16

Data are means ± SE. *$P < 0.01$, compared with control group; †$P < 0.01$, compared with diabetes before treatment; ‡$P < 0.05$, compared with control group.

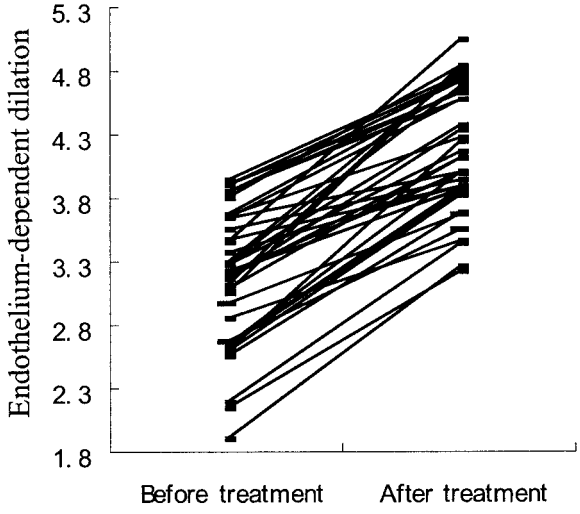

FIG. 2. Changes of endothelium-dependent arterial dilation before and after treatment in diabetic patients.

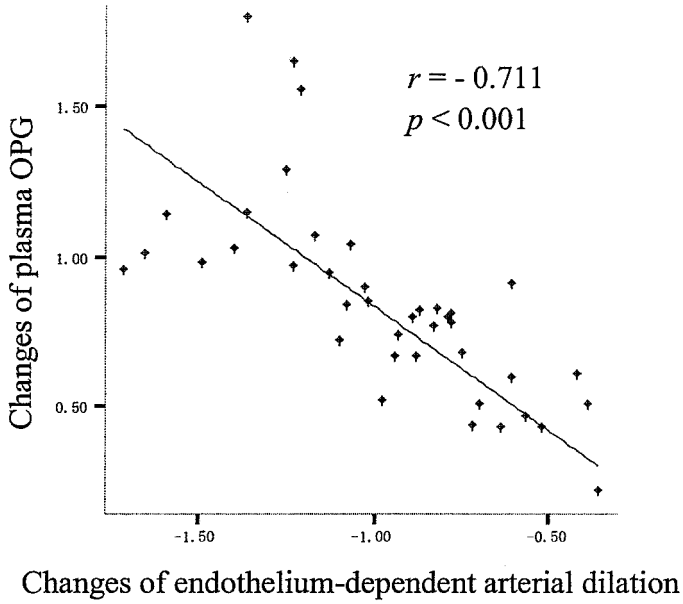

FIG. 3. Spearman's analysis to evaluate correlation of change in osteoprotegerin (OPG) with change in endothelium-dependent arterial dilation before and after treatment in diabetic patients.

vascular calcification induced by treatment with warfarin and vitamin D in a rat model (18).

The significance of osteoprotegerin in human vascular biology has recently been identified by several studies. In a prospective study of almost 500 women, high osteoprotegerin values were associated with increased cardiovascular mortality (10). Two cross-sectional studies of subjects undergoing coronary angiography revealed a strong positive association between osteoprotegerin serum level and advanced coronary artery disease (6,7). Ueland et al. (19) reported that osteoprotegerin is a novel marker of cardiovascular mortality and clinical events in patients with acute myocardial infarction complicated by heart failure. More recently, increased osteoprotegerin plasma levels have been reported in type 2 diabetic patients, and the increased osteoprotegerin levels were associated with microvascular complications (8). Consistent with this, serum osteoprotegerin levels were higher in type 2 diabetic subjects than in nondiabetic subjects, and serum osteoprotegerin levels were significantly associated with inflammation and arterial stiffness (20). Although osteoprotegerin may act as a vascular protective factor, possibly by inhibiting vascular calcification, osteoprotegerin levels appear to be elevated in subjects with vascular damage. Hofbauer and Schoppet (21) therefore proposed that increased osteoprotegerin levels may represent an (incomplete) defense mechanism against other factors that promote arterial calcification, atherosclerosis, and other forms of vascular damage. For many years, it has been known that endothelial dysfunction is an important early event in atherogenesis (11). In the current study, we confirmed elevated osteoprotegerin levels in type 2 diabetic patients. It should be mentioned that our study subjects were newly diagnosed with diabetes, and plasma osteoprotegerin levels are negatively correlated with endothelium-dependent arterial dilation, suggesting that osteoprotegerin might affect early-stage atherosclerosis in diabetic patients.

Hyperglycemia and elevated A1C are cardiovascular risk factors. Here, we found that plasma osteoprotegerin levels are positively correlated with FBG and A1C levels, which are in good agreement with previously published data (20). Consistent with previous findings by Browner et al. (10), we did not find any relation between plasma osteoprote-

TABLE 3
Multiple regression analysis to evaluate association of basal levels of serum osteoprotegerin and other parameters in diabetic patients before therapy

	Model					
Independent variables	1	2	3	4	5	6
Flow-mediated dilation	−0.624*	−0.637*	−0.611*	−0.636*	−0.629*	−0.645*
CRP	0.508*	0.485*	0.521*	0.515*	0.511*	0.506*
FBG	0.314†	0.309†	0.287†	0.296†	0.313†	0.294†
2-h blood glucose	0.137	0.124	0.135	0.128	0.125	0.119
A1C	0.442*	0.456*	0.408*	0.437*	0.419*	0.422*
BMI	—	0.115	—	—	—	—
Mean blood pressure	—	—	0.094	—	—	—
Triglycerides	—	—	—	0.128	—	—
LDL cholesterol	—	—	—	—	0.142	—
Lp (a)	—	—	—	—	—	0.156
r^2	0.545*	0.536*	0.506*	0.541*	0.551*	0.558*

*$P < 0.01$; †$P < 0.05$.

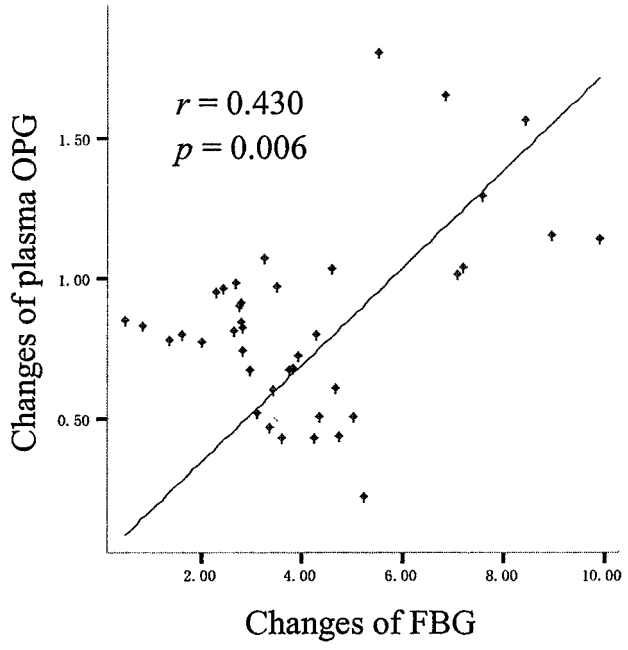

FIG. 4. Spearman's analysis to evaluate correlation of change in osteoprotegerin (OPG) with change in FBG before and after treatment in diabetic patients.

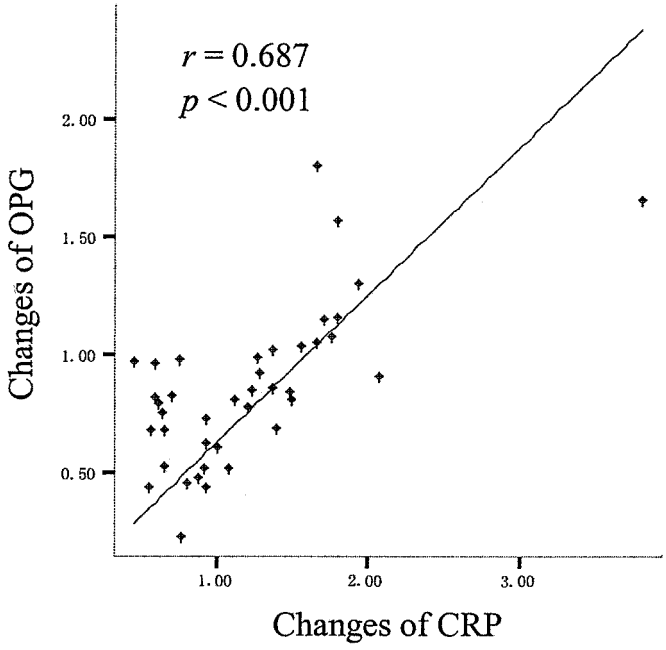

FIG. 6. Spearman's analysis to evaluate correlation of change in osteoprotegerin (OPG) with change in CRP before and after treatment in diabetic patients.

gerin and BMI, total cholesterol, triglycerides, LDL cholesterol, or HDL cholesterol. Recently, Kiechl et al. (16) found that osteoprotegerin levels were related to inflammatory markers such as CRP. More recently, two studies confirmed this association (20,22). In the current study, we also observed the relationship between plasma osteoprotegerin and CRP levels in type 2 diabetic patients, suggesting that plasma osteoprotegerin levels are related to inflammatory markers. In addition, many studies have shown that microalbuminuria is a marker of endothelial dysfunction (23,24). However, we did not find any relation between UAER and endothelium-dependent arterial dilation in the current study. The possible explanation is that the UAERs of all diabetic patients are within normal range.

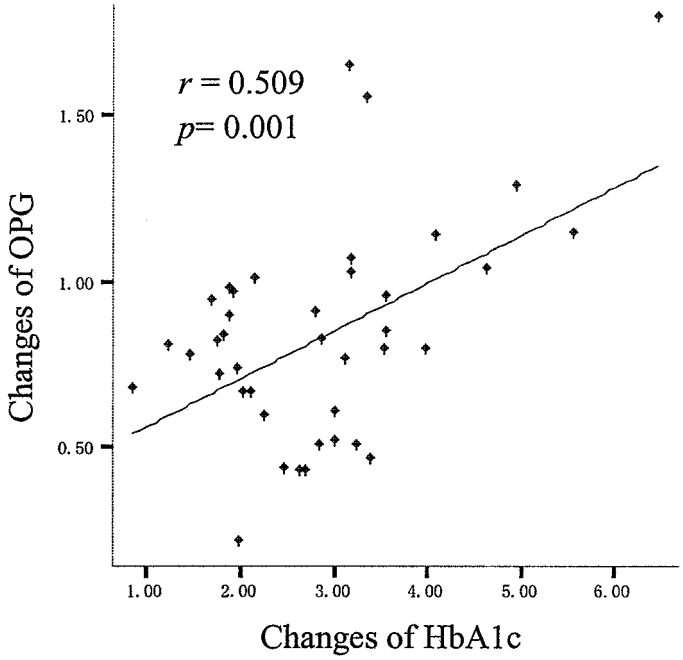

FIG. 5. Spearman's analysis to evaluate correlation of change in osteoprotegerin (OPG) with change in A1C before and after treatment in diabetic patients.

Osteoprotegerin is produced by a variety of cell types, including endothelial cells and smooth muscle cells. Therefore, the origin of the increased plasma osteoprotegerin levels in diabetic subjects is uncertain. We have previously shown that elevated plasma osteoprotegerin levels are associated with endothelial dysfunction in hypothyroidism (25). In the current study, we found that plasma osteoprotegerin showed significant correlation with endothelium-dependent arterial dilation in type 2 diabetic patients. Thus, the elevated osteoprotegerin levels in diabetic patients might represent an increased production of this molecule mainly by endothelial cells.

Some limitations of the current study should be mentioned. First, we did not measure the corresponding levels of RANKL. It has been well established that the ratio of osteoprotegerin to RANKL carries the more relevant information. Second, because bone metabolism markers were not measured, the relation between plasma osteoprotegerin levels and bone metabolism markers could not be evaluated. Further studies are needed to assess this relationship.

In conclusion, this study shows that plasma osteoprotegerin levels are elevated in newly diagnosed diabetic patients and that plasma osteoprotegerin levels are significantly associated with endothelial function. Further studies are warranted to determine the functional role of osteoprotegerin in the development of atherosclerosis in diabetic patients.

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