Effects of Plasma Adiponectin Levels on the Number and Function of Endothelial Progenitor Cells in Patients With Coronary Artery Disease

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Background  It is not known whether plasma adiponectin levels are associated with the number and function of endothelial progenitor cells (EPCs) in patients with coronary artery disease (CAD).

Methods and Results  Plasma levels of adiponectin were measured in 70 patients undergoing coronary angiography. The numbers of colony-forming units (CFUs) of EPCs and senescent EPCs, determined by acidic β-galactosidase staining, were counted. The angiogenic growth factors in the culture medium were also measured. There was a significant positive correlation between adiponectin level and CFUs (r=0.257, p<0.05) but not with the occurrence of senescent EPCs. Next, patients were divided into a high adiponectin group (high ADP: ≥6.17 μg/ml, n=36) and low adiponectin group (low ADP: <6.17 μg/ml, n=34). The number of diseased coronary arteries was less in the high ADP group than that in the low ADP patients (1.7±0.8 vs 2.1±0.7, p<0.05). No significant differences between the 2 groups were demonstrated in angiogenic growth factors secreted from EPCs.

Conclusions  The results suggest that plasma adiponectin levels are associated with the number of EPCs in patients with CAD. (Circ J 2007; 71: 1376–1382)

Key Words: Adiponectin; Coronary artery disease; Endothelial progenitor cells

The integrity and functional activity of the endothelial monolayer play a critical role in atherogenesis. Cardiovascular risk factors induce endothelial injury and a cascade of proinflammatory events, resulting in infiltration of monocytes and smooth muscle cell proliferation, which lead to the formation of atherosclerotic lesions. In fact, the long-term follow-up of patients with endothelial dysfunction suggests that reduced bioavailability of nitric oxide (NO) may even have prognostic implications and contribute to the progression of coronary atherosclerosis. On the other hand, endothelial progenitor cells (EPCs) play a protective role against endothelial damage through repairing injured endothelium and maintaining endothelial function. Clinical studies have demonstrated that the number and/or function of EPCs may be related to the cumulative event-free survival from death from cardiovascular causes and major cardiovascular events. Therefore, the number and function of EPCs are considered to be of clinical importance in a given individual.

The metabolic syndrome is a cluster of generally accepted cardiovascular risk factors, such as impaired glucose metabolism, elevated blood pressure, dyslipidemia, and central obesity. There is increasing evidence that adiponectin, an adipocyte-derived plasma protein, plays an important role in the development of metabolic syndrome, with antiatherogenic properties. Hyperadiponectinemia has been independently associated with the presence of coronary artery disease (CAD) in men, after adjustment for other well-known CAD risk factors. In addition, low plasma levels of adiponectin are associated with vascular endothelial dysfunction in humans. Taken together, these findings indicate that adiponectin may be involved in the pathogenesis of impaired endothelium; however, the mechanisms remain to be fully elucidated.

The hypothesis tested in the present study was that adiponectin is associated with the number and function of EPCs in patients with CAD. To investigate this, we measured the plasma level of adiponectin and evaluated the number and function of EPCs in patients with CAD.

Methods

Patients

Those eligible for entry were 70 patients who underwent coronary angiography at Wakayama Medical University from October 2004 through September 2005 because of an abnormal ECG or angina-like chest pain. Patients with a previous history of coronary intervention or coronary artery bypass graft surgery were excluded to avoid artificial bias from such procedures. We also excluded patients with renal dysfunction (serum creatinin >2.0 mg/dl), malignant disease, and inflammatory disease. None of the patients was taking any type of thiazolidinedione, which is an insulin-sensitizing agent known to increase plasma concentrations of adiponectin. Written informed consent was given by each patient before study participation. The study was approved by the Ethics Review Board of Wakayama Medical University.

Definition of Risk Factors for CAD

Diabetes mellitus was defined according to World Health...
Organization criteria. Dyslipidemia was defined as a total cholesterol concentration >220 mg/dl, triglyceride concentration >150 mg/dl, high-density lipoprotein-cholesterol concentration <40 mg/dl, and/or having received treatment for dyslipidemia. Hypertension was defined as systolic blood pressure >140 mmHg, diastolic blood pressure >90 mmHg or having received treatment for hypertension. Smoking was defined as current smoker. Creatinine clearance (Ccr) was estimated by the Cockcroft-Gault equation: Ccr = (140 – age) × body weight (kg)/72 × serum creatinine (mg/dl).

Isolation and Characterization of EPCs

The peripheral blood mononuclear cell fraction (PB-MNCs) was isolated by density-gradient centrifugation with Histopaque-1077 (Sigma Chemical Co, St Louis, MO, USA) and washed 3 times in Dulbecco's phosphate-buffered saline (GIBCO). After resuspension in endothelial cell basal medium-2 (EBM-2) (Clonetics, Walkersville, MD, USA) containing 5% fetal bovine serum, 0.1% human vascular endothelial growth factor (VEGF)-1, 0.4% human fibroblast growth factor-2, 0.1% insulin-like growth factor, 0.1% human epidermal growth factor, and 0.1% ascorbic acid. To eliminate the possibility of contaminating the assay with mature circulating endothelial cells, we performed an initial preplating step in a fibronectin-coated 6-well plate using 5×10⁶ PB-MNCs per well. After 48 h, the nonadherent cells were collected and 1×10⁶ cells were replated onto fibronectin-coated 24-well plates for a final assessment of the number of colonies. Growth medium was changed every 3 days and the numbers of colonies were counted 7 days after plating. Plates were studied under phase-contrast microscopy, and colonies were counted by 2 independent investigators. A colony of EPCs consisted of multiple thin, flat cells emanating from a central cluster of rounded cells (Fig 1A). A central cluster alone without associated emanating cells was not counted as a colony. Confirmation of endothelial-cell lineage was performed as previously described. Briefly, indirect immunostaining was performed using endothelial-specific antibodies directed against VEGF receptor 2 and CD31 or 1,1'-dioctadecyl-3,3,3',3'-tetramethyl indocarbocyanine-labelled acetylated low-density lipoprotein and costaining with fluorescein isothiocyanate-labeled Ulex europaeus agglutinin 1 (UEA-1, Sigma Chemical Co) (Fig 1B).

Acidic β-Galactosidase Staining

For the measurement of cellular senescence, cultures of EPCs were maintained for 7 days, and senescence-associated β-galactosidase activity was measured as previously described. Cells were counterstained with 4',6-diamidino-phenylindole (DAPI; 0.2 μg/ml in 10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L EDTA, 100 mmol/L NaCl) for 10 min to count the total number of cells. Only cells with a distinctly blue cytoplasm, indicating β-galactosidase activity, were counted. The percentage of β-galactosidase-positive cells for DAPI-positive cells was determined by counting 4 random fields under microscopy (×20), which contained a total of approximately 100–200 cells.

Growth Factor Secretion

To assess the paracrine effects of EPCs, we evaluated the angiogenic cytokines secreted by them. EPCs at day 7 were switched to growth-factor-free medium EBM-2 with 5% FBS for 72 h. Conditioned media was separated and stored at –80°C until measurement. They were assayed for the angiogenic growth factors, VEGF, basic-fibroblast growth factor (b-FGF), hepatocyte growth factor (HGF), and macrophage chemotactic protein-1 (MCP-1) to assess the paracrine effects of EPCs. The angiogenic growth factors and chemokines were assayed by enzyme-linked...
Immunoassay (ELISA) (R&D Systems, Inc, Minneapolis, MN, USA).

Plasma Adiponectin

Venous blood samples were obtained just before coronary angiography and the plasma samples were immediately stored at −80°C for subsequent assay. Plasma levels of adiponectin were measured with a quantitative ELISA technique (R&D Systems). Samples were checked by serial dilution, and measurements were performed in duplicate as a minimum.

Coronary Angiography

Two experienced observers, unaware of all patient characteristics, independently reviewed all coronary angiograms. By visual assessment, the number of diseased coronary arteries was defined (1-, 2-, or 3-vessel disease). A reducing stenosis ≥50% diameter was regarded as significant. In addition, the extent of coronary stenosis was assessed using the scoring system previously described by Sullivan et al. The length proportion of each vessel involved by angiographically detectable atheroma was evaluated and multiplied by a factor for each vessel: left main system, 5; left anterior descending artery, 20; main diagonal branch, 10; first septal perforator, 5; left circumflex artery, 20; obtuse marginal and posterolateral vessels, 20; and main posterior descending branch, 10. The scores for each vessel or branch were added to give a total score up to a maximum of 100, representing the percentage of the coronary luminal surface area involved by atheroma, as described in previously.

Statistical Analysis

Results of normally distributed continuous variables are expressed as the mean±SD, and those for continuous variables with skewed distribution as the median value. Comparison of continuous variables was analyzed with the unpaired t-test and Mann-Whitney U-test, as appropriate. Associations between the number of either EPC colonies or plasma adiponectin levels and all parameters were first

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**Table 1 Multivariate Regression Analysis of Variables Associated With CFUs of EPCs in Study Subjects**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Standard coefficient</th>
<th>F</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>−0.263</td>
<td>3.585</td>
<td>0.063</td>
</tr>
<tr>
<td>Male</td>
<td>−0.178</td>
<td>1.539</td>
<td>0.220</td>
</tr>
<tr>
<td>Body mass index</td>
<td>−0.070</td>
<td>0.190</td>
<td>0.664</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.115</td>
<td>0.599</td>
<td>0.442</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>−0.051</td>
<td>0.132</td>
<td>0.717</td>
</tr>
<tr>
<td>Dyslipidemia</td>
<td>−0.005</td>
<td>0.001</td>
<td>0.976</td>
</tr>
<tr>
<td>Family history of CAD</td>
<td>0.092</td>
<td>0.435</td>
<td>0.512</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.039</td>
<td>0.080</td>
<td>0.779</td>
</tr>
<tr>
<td>Statin use</td>
<td>−0.127</td>
<td>0.594</td>
<td>0.444</td>
</tr>
<tr>
<td>ACEI/ARB use</td>
<td>0.117</td>
<td>0.662</td>
<td>0.419</td>
</tr>
<tr>
<td>Log adiponectin</td>
<td>0.321</td>
<td>4.991</td>
<td>0.029</td>
</tr>
</tbody>
</table>

**Table 2 Clinical Characteristics of Patients With High and Low Plasma Adiponectin Levels**

<table>
<thead>
<tr>
<th></th>
<th>High plasma adiponectin (n=34)</th>
<th>Low plasma adiponectin (n=36)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>70.0±7.3</td>
<td>67.4±9.2</td>
<td>0.186</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>25 (73.5)</td>
<td>34 (94.4)</td>
<td>0.019</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>22.8±3.3</td>
<td>24.9±2.5</td>
<td>0.005</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>24 (70.1)</td>
<td>30 (83.3)</td>
<td>0.213</td>
</tr>
<tr>
<td>Diabetes, n (%)</td>
<td>9 (26.5)</td>
<td>15 (41.7)</td>
<td>0.184</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>21 (61.8)</td>
<td>23 (63.9)</td>
<td>0.857</td>
</tr>
<tr>
<td>Smoking, n (%)</td>
<td>18 (52.9)</td>
<td>27 (75.0)</td>
<td>0.056</td>
</tr>
<tr>
<td>Family history of CAD, n (%)</td>
<td>7 (20.1)</td>
<td>14 (38.9)</td>
<td>0.096</td>
</tr>
<tr>
<td>Log adiponectin, µg/ml</td>
<td>1.05±0.15</td>
<td>0.50±0.20</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

Data are mean±SD, or number (%), as appropriate. Abbreviation see in Table 1.
Fig 3. Relationship between plasma adiponectin levels and the severity of coronary artery disease. (A) The 70 subjects were divided into 4 groups according to the extent score: <25, 26–50, 51–75, >75. Plasma adiponectin levels were significantly lower in groups with an extent score >51 than in groups with a score <50. (B) The 70 subjects were divided into 2 groups according to plasma adiponectin level: below-median adiponectin (Low ADP) group, and above-median adiponectin (High ADP) group. The number of diseased coronary arteries was significantly higher in the high ADP group than in the low ADP group. Colony-forming units were significantly higher in the high ADP group than in the low ADP group. (C) The 70 subjects were divided into 4 groups according to the number of diseased vessels: none (0), 1 vessel (1-VD), 2 vessels (2-VD), and 3 vessels (3-VD). Plasma adiponectin levels were significantly lower in the groups with more than 2-vessel disease than those with less than 1-vessel disease. Creatinine clearance (Ccr) levels were significantly correlated with the subjects’ log-transformed adiponectin levels (D), but no significant correlation was found between left ventricular ejection fraction (EF) and log-transformed adiponectin levels (E).
analyzed by simple logistic regression analysis and then by multivariate analysis. Statistical significance was defined as p<0.05. All analysis were performed using Stat View-5.0 software (SAS Institute Inc, Cary, NC, USA) and SPSS 14.0J for Windows (SPSS Inc, Tokyo, Japan).

Results

Plasma Adiponectin Levels and Number of EPCs

Because the distribution of plasma adiponectin levels in the present study subjects was relatively close, we adopted the log-transformed plasma adiponectin (plasma log-adiponectin) levels, not the raw plasma adiponectin levels as in the previously reported correlation studies.\(^8,10\) A significant positive correlation was found between log-transformed plasma adiponectin levels and the number of colony-forming units (CFUs) of EPCs (\(r=0.257, p=0.016\)) (Fig 2A). In contrast, no significant correlation was found between plasma adiponectin levels and the occurrence of senescent EPCs (Fig 2B). Multiple regression analysis revealed that only plasma adiponectin levels were significantly associated with the number of EPCs (Table 1).

Plasma Levels of Adiponectin in Patients With CAD

We divided the 70 patients into 2 groups using the median value of the plasma adiponectin level (6.17 pg/ml). With this definition, 36 patients were classified into the below-median adiponectin (low ADP) group, and 34 into the above-median...
adiponectin (high ADP) group because there were 2 patients with a plasma adiponectin level of 6.17 μg/ml. The clinical characteristics of the patients are shown in Table 2. The number of men and the body mass index were significantly lower in patients with high ADP than in those with low ADP. When we examined the relationship between the extent of coronary stenosis (extent score) and plasma adiponectin levels, we found that the higher the extent score, the lower the plasma adiponectin level (Fig 3A). Next, we investigated the relationship between the number of diseased vessels and plasma adiponectin levels. The number of diseased vessels in low ADP group was significantly higher than that in the high ADP group (Fig 3B). We also found that plasma adiponectin levels were significantly lower in those with more than 2-vessel disease compared with those with less than 1-vessel disease (Fig 3C). In addition, a significant positive correlation was found between log-transformed plasma adiponectin levels and Ccr (Fig 3D). Finally, no significant correlation was found between log-transformed plasma adiponectin levels and left ventricular ejection fraction (Fig 3E).

**Number of EPCs and Severity of CAD**

When we examined the relationship between the number of CFUs of EPCs and the extent score, we did not find any significant relationship between them (Fig 4A). Similarly, we did not find any significant relationship between the number of EPCs and the number of diseased vessels (Fig 4B).

**Plasma Adiponectin Levels and Angiogenic Growth Factors**

Because EPCs are reportedly a source of angiogenic growth factors, which may contribute to angiogenesis of adjacent endothelial cells in a paracrine manner, we evaluated the angiogenic cytokines secreted by EPCs. Over a 72-h period, measurable amounts of VEGF, b-FGF, HGF and the angiogenic cytokines secreted by EPCs.11,12 Recently, eNOS-derived NO was shown to promote the release of EPCs from the bone marrow through nitrosylation and activation of matrix metalloproteinase-9 and increased expression of VEGF. VEGF, HMG-CoA reductase inhibitors (statins),13 estrogen,13 and erythropoietin14 appear to share a common phosphatidylinositol 3 (PI3K)/Akt signaling pathway in the regulation of EPC kinetics, leading to increased EPCs. Interestingly, it has been revealed that adiponectin exerts its vascular actions by direct stimulation of NO production in endothelial cells using phosphorylation of eNOS by AMP-activated protein kinase (AMPK).15 In addition, in mouse and rabbit models of angiogenesis, adiponectin induces endothelial cell differentiation and migration with blood vessel development by promoting cross-talk between AMPK and Akt kinase signaling within the endothelial cells.16 Taken together, the findings suggest that adiponectin might mobilize EPCs from bone marrow in a NO-dependent manner. Another possibility is that adiponectin might play a role in an anti-apoptotic effect on circulating EPCs. In fact, adiponectin inhibits apoptosis of endothelial cells through an AMPK-dependent mechanism.17 Because EPCs senescence determined by β-galactosidase activity appears to be associated with cellular apoptosis,18 we investigated the relationship between the plasma level of adiponectin and EPCs senescence. However, in our study population no significant relation was demonstrated between plasma adiponectin levels and EPCs senescence. Therefore, the former explanation is plausible. Although the precise mechanisms remain unsolved, the present study extends the previous findings by demonstrating that adiponectin increases the number of circulating EPCs.

Endothelial damage represents a balance between the magnitude of injury and the capacity for repair. Given that EPCs play a role in maintaining endothelial function through endothelial repair, further understanding of the mechanisms that regulate EPCs biological activity may provide new insights into the pathogenesis of vascular genenesis. Here, we have demonstrated for the first time that the plasma level of adiponectin is significantly associated with an increase in the number of circulating EPCs in patients with CAD, and in our study population the plasma level of adiponectin was an independent predictor of the number of EPCs.

The mechanisms of the positive relationship between the plasma adiponectin level and the number of EPCs remain to be determined. One possible explanation is that adiponectin promotes EPC mobilization from bone marrow and differentiation of CD34+ hematopoietic precursor cells into EPCs. It is well established that the serine/threonine kinase Akt-mediated endothelial NO synthase (eNOS) pathway plays a central role, not only in mature endothelial cells, but also in EPCs.11,12 Recently, eNOS-derived NO was shown to promote the release of EPCs from the bone marrow through...
tective mechanisms against atherogenesis, to our knowledge the present study represents the first report demonstrating that low adiponectin levels are significantly associated with the number of EPCs, possibly reflecting the maintenance of endothelial function.

Study Limitations
First, the relatively small number of patients studied. We demonstrated that the plasma adiponectin level is an independent predictor of the number of EPCs in patients with CAD in multiple regression analysis, though it was barely significant. Further studies in a large number of patients should confirm our results.

In conclusion, plasma adiponectin levels might be related to the number of circulating EPCs in patients with CAD.

References