Clinical Characteristics of Patients With Kawasaki Disease and Levels of Peripheral Endothelial Progenitor Cells and Blood Monocyte Subpopulations

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Background: Coronary sequelae that persist after Kawasaki disease (KD) have been associated with coronary vascular events in adolescents and young adults. The aim of this study was to investigate the relationship between coronary sequelae late after KD and circulating endothelial progenitor cells (EPCs), as a marker of vascular repair, or monocyte subsets as a marker of inflammation.

Methods and Results: The 31 KD patients were divided into 3 groups according to the type of coronary artery lesion (CAL): group 1 consisted of 14 patients with persistent aneurysm; group 2 consisted of 9 patients with regressed aneurysms; group 3 included 8 KD patients with normal coronary arteries from disease onset. The control group (group 4) consisted of 10 healthy subjects. Flow cytometric analysis was used to quantify circulating EPCs defined as CD34+KDR+ cells and 2 distinct monocyte subsets (CD14+CD16+ and CD14+CD16–). The number of EPCs in group 1 and group 2 was significantly decreased compared with group 4. In contrast, neither the number of CD14+CD16+ monocytes nor that of CD14+CD16– monocytes was significantly different among the 4 groups. Finally, there were not any significant relationship between the numbers of EPCs and the 2 monocyte subsets.

Conclusions: There are lower numbers of EPCs in the chronic phase of KD, irrespective of both CAL formation and monocyte subsets. (Circ J 2010; 74: 2720–2725)

Key Words: Coronary artery lesions; Endothelial progenitor cells; Kawasaki disease; Monocyte subsets

Kawasaki disease (KD) is an acute febrile disorder with coronary and other systemic vasculitides that occurs predominantly in infancy and early childhood. Later in adulthood, angiographic and postmortem studies have demonstrated that coronary sequelae persisting long after acute KD vasculitis predispose to atherosclerotic changes, which are associated with late coronary events or sudden death. However, the mechanisms underlying late coronary vascular disease are poorly understood. Because vessel wall homeostasis is a fine balance between the defense mechanisms of repair and injury induced by inflammation, in the present study we examined both endothelial progenitor cells (EPCs), as a marker of vascular repair, and monocyte subsets as a marker of inflammation.

One of the most fascinating breakthroughs in the field of vascular biology in the past few decades was the discovery of EPCs. These cells are mobilized from the bone marrow into the peripheral circulation, and may contribute to postnatal blood vessel repair and neovascularization. Clinically, the number of EPCs is lower in patients with cardiovascular risk factors and/or established atherosclerosis. In addition, their putative role in vascular biology has been further supported by 2 recent studies demonstrating that a reduction in the number of EPCs is an independent predictor of future cardiovascular events.

On the other hand, recent studies have also shown that monocytes in peripheral blood are heterogeneous. Differential expressions of CD14 and CD16 allow monocytes to be divided into 2 subsets: CD14+CD16+ and CD14+CD16–. Distinct chemokine-receptor expression profiles are also among the phenotypic differences recognized between the subsets: for example, CD14+CD16+ monocytes express the C-C motif chemokine receptor 2, whereas CD14+CD16+ monocytes express the C-X3-C motif chemokine receptor 1.

We hypothesized that the persistence of coronary artery lesions (CALs), especially coronary aneurysms, late after KD...
is associated with both impaired endothelial repair and augmentation of specific monocyte subsets. In this study, we therefore investigated the numbers of circulating EPCs and 2 distinct monocyte subsets in referents and patients with and without CALs late after KD.

**Methods**

**Study Subjects**

The patients who had had acute KD more than 1 year previously were enrolled. All KD patients met the diagnostic criteria established by the Japanese Kawasaki Disease Research Committee; 31 patients (21 males) with a mean age of 154±69 months (range 31–322 months) were studied 113±71 months (range 13–234 months) after acute KD, which had occurred at 41±38 months of age (range 2–131 months). During the acute stage, 23 patients with KD had dilation of the proximal left anterior descending and/or right coronary arteries, with a diameter greater than 3.5 mm; 14 patients still had dilated coronary arteries >3.5 mm at the time of the present study (group 1) and the dilated coronary arteries had regressed during follow-up in 9 patients (group 2) (Table 1). The initial diameter in the patients in group 1 was significantly larger than that in group 2 (8.2±3.3 mm and 5.1±1.1 mm, respectively, P<0.01). Group 3 consisted of 8 patients with KD who had no CALs from disease onset (Table 1). The control group (group 4) consisted of 10 healthy age- and sex-matched subjects (mean age: 206±29 months, range: 13–228 months) (Table 1). Of the 14 patients in group 1, 9 had giant coronary aneurysms, and 5 had persistent small coronary aneurysms. The KD patients with CAL in the acute phase had been administered 1 or several antiplatelet agents: 17 took aspirin (group 1: n=14; group 2: n=3), 7 took clopidogrel (group 1: n=7), and 3 took dipyridamole (group 1: n=2, group 2: n=1). 7 patients in group 1 had received both antiplatelet therapy and warfarin for coronary thrombus. All patients were free of symptoms during their daily activities. All of the total 31 patients with KD had been treated with various doses of intravenous immunoglobulin during the acute phase of illness. Healthy (control) subjects were normal children without risk factors for endothelial damage and were recruited from family and friends of hospital staff. None was taking medication or had a history of significant illness.

The study protocol was approved by the Ethical Committee of Wakayama Medical University, and written informed consent was given by each patient and/or parents.

**Quantification of Circulating EPCs and Monocyte Subsets by Flow Cytometry**

To quantify circulating EPCs, immunofluorescent cell staining was performed using fluorescent conjugated antibody CD34-fluorescein isothiocyanate (FITC) (10 μg; Becton Dickison), and kinase insert domain receptor (KDR). The EPC count by flow cytometry was performed with fresh isolated peripheral blood mononuclear cells (PBMCs) using FITC (10 μg; Becton Dickison), and KDR. PBMCs were 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). For the identification of KDR+ cells, indirect immunolabeling was performed with the use of a biotinylated goat mononuclear antibody against the extracellular domain of human KDR (R&D Systems). IgG2a-FITC-PE antibody (Becton Dickison) served as a negative control. For staining of KDR, extensive blocking was required with the use of human immunoglobulin (polyglobulin, 10%; Bayer) and goat serum (Sigma-Aldrich). A total of 150 μl of PBMCs was incubated for 15 min at room temperature. Cell fluorescence was measured immediately after staining, and data were analyzed using CellQuest software (FACSCalibur, Becton Dickison). Units of all measured components are absorbed cell counts obtained after the measurement of 10,000 events in the lymphocyte gate. For each patient, a corresponding negative control with IgG2a-FITC-PE antibody was obtained. The frequency of PBMCs positive for CD34 and KDR was determined by a 2-dimensional side-scatter fluorescence dot-plot analysis of the sample, after appropriate gating, stained with different reagents.

To quantify the monocyte subsets, monoclonal antibodies against CD14 (FITC-conjugated, Clone M5E2; BD Bioscience) and CD16 (PE-Cy5-conjugated, Clone 3G8, BD Bioscience) were used as previously reported. Matched isotype controls (FITC Mouse IgG2a isotype control and PE-Cy5 Mouse IgG1 isotype control) purchased from the same company were used as negative controls. A total of 100 μl of blood was left to stand for 15 min at room temperature. For erythrocyte lysis and leukocyte fixation, 1 ml of lysing solution was added (BD FACS Lyse, Becton Dickison, Germany).

Cytometric analysis was performed in a flow cytometer (FACSCalibur, Becton Dickison) using Cell Quest Software Systems (Becton Dickison). Monocytes were first gated in a forward scatter/side scatter dotplot, and then FITC- and PE-channel fluorescence was measured within the monocyte gate. CD14+CD16 cells were defined as monocytes expressing CD14, but not CD16, while CD14+CD16 cells were defined as monocytes expressing CD16 and either high levels of CD14 (CD14intCD16+) or low levels of CD14 (CD14dimCD16+). Thus, CD14intCD16+ and CD14dimCD16+ were not analyzed separately, as in a previous study.

**Biochemical Measurements**

Serum total cholesterol, triglycerides, high-density lipoprotein cholesterol, and fasting glucose were determined by standard laboratory methods. The concentration of low-density lipoprotein cholesterol was calculated by Friedewald’s equation. The level of high-sensitivity C-reactive protein (hsCRP) was analyzed using a commercially available testing kit (N-Latex CRP II, Dade Behring, Marburg, Germany).

**Statistical Analysis**

All statistical analyses were performed with the statistical software package SPSS version 16.0 (SPSS, Chicago, IL, USA). Data are presented as mean±SD, unless stated otherwise. For the analysis of clinical characteristics, the significance of any difference in means among 3 or 4 groups was tested with a 1-way ANOVA, followed by Scheffe’s F test; differences in proportions were tested with Fisher’s exact test. Because the number of CD34+KDR+ cells and the frequency
of CD14+CD16+ monocytes were not normally distributed, they are expressed as median and range, and the significance of any differences among the 4 groups was assessed by Kruskal-Wallis test. If a significant difference was found, pairwise comparison by Bonferroni test was performed for multiple analysis. Statistical significance was accepted if the null hypothesis could be rejected at P<0.05.

Results

Patients’ Characteristics and Clinical Data

The detailed clinical characteristics of the study population are summarized in Table 2. There were no significant differences in age, sex distribution, body mass index, blood pressure, lipid profiles or hsCRP level among the 4 groups.

Table 2. Clinical Characteristics of the Study Population

<table>
<thead>
<tr>
<th></th>
<th>Group 1 (n=14)</th>
<th>Group 2 (n=9)</th>
<th>Group 3 (n=8)</th>
<th>Group 4 (n=10)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at study, months</td>
<td>172±88</td>
<td>139±47</td>
<td>142±46</td>
<td>206±29</td>
<td>0.08</td>
</tr>
<tr>
<td>Interval, months</td>
<td>112±88</td>
<td>125±51</td>
<td>103±64</td>
<td>NA</td>
<td>0.82</td>
</tr>
<tr>
<td>Male, n (%)</td>
<td>8 (57)</td>
<td>8 (89)</td>
<td>5 (63)</td>
<td>7 (70)</td>
<td>0.47</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>18.1±2.4</td>
<td>18.2±3.3</td>
<td>20.1±4.4</td>
<td>20.8±2.2</td>
<td>0.12</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>108±10</td>
<td>109±11</td>
<td>111±9</td>
<td>106±8</td>
<td>0.69</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>63±7</td>
<td>59±11</td>
<td>60±8</td>
<td>57±8</td>
<td>0.42</td>
</tr>
<tr>
<td>TC, mg/dl</td>
<td>167±26</td>
<td>159±25</td>
<td>177±33</td>
<td>177±34</td>
<td>0.5</td>
</tr>
<tr>
<td>TG, mg/dl</td>
<td>83±58</td>
<td>79±43</td>
<td>69±23</td>
<td>70±37</td>
<td>0.85</td>
</tr>
<tr>
<td>HDL-C, mg/dl</td>
<td>59±13</td>
<td>52±7</td>
<td>59±9</td>
<td>66±12</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL-C, mg/dl</td>
<td>91±18</td>
<td>92±19</td>
<td>104±28</td>
<td>97±29</td>
<td>0.6</td>
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<tr>
<td>hsCRP, mg/L</td>
<td>0.37±0.41</td>
<td>0.37±0.40</td>
<td>0.28±0.22</td>
<td>0.24±0.23</td>
<td>0.78</td>
</tr>
<tr>
<td>Blood white cells, /μl</td>
<td>6,031±1,551</td>
<td>6,533±1,670</td>
<td>5,610±1,058</td>
<td>5,860±1,764</td>
<td>0.65</td>
</tr>
<tr>
<td>Mononuclear cells, /μl</td>
<td>2,438±744</td>
<td>2,307±605</td>
<td>2,640±710</td>
<td>2,242±556</td>
<td>0.64</td>
</tr>
<tr>
<td>Monocytes, /μl</td>
<td>256±135</td>
<td>326±192</td>
<td>308±105</td>
<td>269±116</td>
<td>0.63</td>
</tr>
<tr>
<td>Lymphocytes, /μl</td>
<td>2.182±791</td>
<td>2.048±535</td>
<td>2.338±759</td>
<td>1.973±502</td>
<td>0.68</td>
</tr>
<tr>
<td>CD14+CD16− monocytes, /μl</td>
<td>214±106</td>
<td>282±82</td>
<td>256±83</td>
<td>226±103</td>
<td>0.57</td>
</tr>
<tr>
<td>CD14+CD16+ monocytes, /μl</td>
<td>28±28</td>
<td>26±14</td>
<td>30±21</td>
<td>27±9</td>
<td>0.99</td>
</tr>
</tbody>
</table>

Group 1 consisted of patients with persistent aneurysm. Group 2 consisted of patients with regressed aneurysms. Group 3 was patients with normal coronary arteries from disease onset. Group 4 consisted of control (healthy) subjects. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein.

Figure 1. Representative dot plots of flow cytometry of endothelial progenitor cells according to the expression cell surface antigens, such as CD34 and kinase domain receptor (KDR). Representative dot plots from groups 1 (A) and 4 (B). (A) Dots in right upper indicate the CD34+/KDR+ cells among peripheral blood mononuclear cells (PBMCs) from a patient with persistent aneurysm (146 month, male). (B) Dots in right upper indicate the CD34+/KDR+ cells among PBMCs from a healthy (control) subject (266 month, male).
The initial vessel diameter in group 1 patients was significantly larger than that in group 2 (8.2±3.3 mm and 5.1±1.1 mm, respectively, P<0.01). To assess the effect of circulating EPCs on the fate of CALs in KD patients, we selected 2 groups [group 1 (n=6) and group 2 (n=9)] with similar vessel size (5.6±0.9 mm vs 5.1±1.1 mm, respectively, P=0.35) in the acute phase; there were no significant differences in circulating EPCs numbers between these selected groups (20±6 vs 19±11, respectively, P=0.86), suggesting that CAL size in the acute phase, but not the number of circulating EPCs in the chronic phase, may be a key factor in predicting the fate of CALs in patients with KD.

**EPC Numbers in KD**

Dot plot analyses of typical patients in groups 1 and 4 are shown in **Figure 1**. Numbers of circulating EPCs were defined by the number of CD34+/KDR+ cells and were significantly lower in group 1 and 2 patients compared with group 4 (control group) (**Figure 2**).

**Natural Profile of Monocyte Subsets Levels in KD**

Samples of peripheral whole blood for analysis of the 2 monocyte subsets (CD14+CD16- and CD14+CD16+) were obtained from the 4 groups of patients and there were no significant differences among them (data not shown). Similarly, there were not any significant differences in circulating CD14+CD16- monocytes among the 4 groups (data not shown). In addition, there was not a significant relationship between circulating EPCs and CD14+CD16- monocytes.

**Discussion**

The present study investigated the numbers of circulating EPCs and 2 distinct monocyte subsets in patients with KD. Our data show for the first time that KD patients with CALs, irrespective of the persistence of coronary aneurysms, have reduced numbers of circulating EPC, but not of the circulating monocyte subsets.

EPCs in postnatal bone marrow and peripheral blood may contribute to endothelial repair and neovascularization. Nakatani et al reported that the number of EPCs increased in the acute stage of KD in patients with CAL,19 which is contrary to very recent findings in the convalescent phase.20 This apparent contradiction seems to suggest that EPC mobilization may be biphasic, with an increase and decrease in the acute and convalescent phases, respectively. In the early phase, EPCs are increased, reflecting the bone marrow response to diffuse and severe endothelial damage. In the convalescent phase, increased consumption of EPCs may contribute to decreased circulating EPCs. It is generally believed that reduced numbers of EPCs may be a result of exhaustion of the pool of progenitor cells available in the bone marrow.8,21 Liu et al demonstrated that KD patients with CALs have decreased numbers of circulating EPCs, as defined by expression of both CD133 and KDR, in the late convalescence period.20 The present study has expanded the previous study by demonstrating that the number of circulating EPCs, which was defined by the expression of both CD34 and KDR, in the chronic phase was similar in KD patients with persistence of CAL and in those with regression of CAL. Although recent review articles stated that CD34+KDR+ cells, not CD133+KDR+ cells, are preferred in studies in which the EPC count is intended to be a cardiovascular marker,22,23 more than 50% of CD133+ cells are also positive for CD34. Furthermore, not only CD34+ cells but also CD133+ cells are capable of differentiating into both endothelial and hematopoietic lineage cells. Taking all the findings together, it remains unclear which surface markers are prototypical for the EPC phenotype by means of flow cytometry analysis. Further studies of this important subject are needed.

Importantly, CD34+KDR+ cells have been shown to correlate with cardiovascular risk and subclinical atherosclerosis better than any other antigenic combination and have also been demonstrated repeatedly and convincingly to be an independent predictor of cardiovascular outcome.9-12 All the data indicate that the CD34+KDR+ phenotype represents the best compromise in terms of biological meaning and clinical usefulness.

Early studies of monocyte subpopulations revealed smaller and denser cells (CD14+CD16 monocytes), which might be more potent antigen-presenting cells and a more mature type of monocyte than regular monocytes (CD14+CD16 monocytes).24,25 Moreover, CD14+CD16+ monocytes exhibit higher levels of very late antigen 4, the ligand for vascular cell adhesion molecule-1 and CD11a and c, which binds to intercellular adhesion molecule-1, so these cells may have a greater tendency to adhere to the endothelium and migrate into tissues.26 Katayama et al demonstrated an increase in the number of CD14+CD16+ monocytes in KD patients during the acute stage and also showed a positive correlation between CD14+CD16+ monocytes and C-reactive protein levels during the acute stage.26 Their results suggest that the percentage of the CD14+CD16+ monocyte subpopulation reflects the severity of KD, but on the other hand, our study showed that in patients late after KD there were not any significant relationships between the persistence of CALs and the numbers of the monocyte subsets, suggesting that there is no evidence of inflammation in peripheral blood in terms of...
of these monocyte subsets.

**Clinical Implications**

Our results indicate lower numbers of EPCs in the chronic phase of KD, irrespective of either CAL formation or monocyte subsets. The role of EPCs as a marker of vascular health and prognosis is already well established. More importantly, EPCs might not be simply a marker of vascular health, but might also contribute to vascular health. KD patients with CAL in the acute phase, even if there is regression of the aneurysms in the chronic phase, should be managed thoroughly to prevent cardiovascular diseases. The use of 3-hydroxy-3-methylglutaryl-coenzyme A reductase inhibitors, angiotsin-converting enzyme inhibitors, and angiotensin-receptor blockers may mobilize EPCs and promote vascular wall remodeling. Therefore, measuring the number of circulating EPCs might be clinically useful for evaluating and preventing atherosclerotic change, although there are no data to support this in the setting of KD.

**Study Limitations**

First, this was a single-center study with a small number of patients, and thus the potential risk of patient selection bias. Second, it did not investigate the dynamics of circulating EPCs and thus cannot report on whether differences reflect alterations in EPC mobilization, survival, or tissue uptake. Third, some of the drugs administered to the patients may potentially affect the levels of EPCs and monocyte subsets.

Fourthly, in this study, the frequency of PBMCs positive for CD34 and KDR was determined by a 2-dimensional scatter fluorescence dot-plot analysis of the sample, after appropriate gating, stained with different reagents, as previously reported. However, when performing flow cytometry on fresh peripheral blood, the rarity of circulating EPCs imposes the use of a very limited number of surface antigens. Therefore, assuming that a phenotype based on 2–3 antigens definitely identifies a cell population with a complex function is virtually impossible. Despite these important limitations, flow cytometry is currently the best method of obtaining pure quantitative data on putative EPCs. Being sensitive, specific and reproducible, flow cytometry should be considered the gold standard when peripheral blood count is used as a disease biomarker. Finally, although this study suggests that circulating EPCs may pathophysiologically contribute to endothelial repair in the chronic phase of KD, we could not show a difference in the serial changes in circulating EPCs between groups because of a lack of the data from the acute phase of KD.

**Conclusion**

Our results indicate lower numbers of EPCs in the chronic phase of KD, irrespective of both CAL formation and monocyte subsets. The current strategy of long-term treatment with antiplatelet agents and angioplasty may not be sufficient to arrest the coronary sequelae process. A better understanding of the basic mechanism of coronary artery remodeling may lead to more innovative and effective treatments, such as the pharmacological modulation of EPCs.

**Acknowledgment**

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