Systemic Production of Vascular Endothelial Growth Factor and \textit{fms}-Like Tyrosine Kinase-1 Receptor in Acute Kawasaki Disease

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Background—Increased vascular permeability is an important event during the initial process of Kawasaki disease (KD). One potential responsible candidate for the induction of vascular hyperpermeability is vascular endothelial growth factor (VEGF).

Methods and Results—We investigated the expression of VEGF and its receptors (flt-1, KDR) in acute KD tissues at 7 days to 5 weeks of illness. Neuropilin-1, which enhances the binding of VEGF$_{165}$ to KDR, was also studied. Abundant expression of VEGF and flt-1 was documented immunohistochemically in many organs from acute KD, including heart and lung. VEGF and flt-1 were colocalized in all vessels that showed edema. These molecules resided in endothelium and vascular media and also in migrating smooth muscle cells in neointima and infiltrating macrophages. Compared with controls, coronary vessels of acute KD had upregulation of VEGF and flt-1 but not KDR or neuropilin-1. KDR was expressed by vessels at 7 days of illness but not later in the illness. Plasma proteins were more extensively bound to the extracellular matrix in coronary vessels in acute KD than controls. Furthermore, elevation of serum VEGF levels was correlated with low serum albumin in acute KD ($n=220$, $r=-0.53$, $P<0.001$).

Conclusions—These findings suggest that VEGF and flt-1 are upregulated in blood vessels in many organs of acute KD. Expression of KDR was limited to the early stage of acute KD. The roles of VEGF in acute KD may involve promotion of vascular permeability and macrophage activation. Low serum albumin may indicate overproduction of VEGF in acute KD. (\textit{Circulation}. 2002;105:766-769.)

Key Words: Kawasaki disease \textbullet growth substances \textbullet receptors

Kawasaki disease (KD) is a systemic vasculitis with microvascular hyperpermeability. Electron microscopy of skin biopsy samples in KD demonstrates gap formation and fenestration of endothelial cells, inducing perivascular edematous changes. The molecules responsible for inducing the microvascular hyperpermeability of KD have not been identified conclusively. One candidate is vascular endothelial growth factor (VEGF), which promotes microvascular permeability. This heparin-binding glycoprotein is also mitogenic and angiogenic for endothelial cells. VEGF exerts its biological functions through high-affinity tyrosine kinase receptors, the \textit{fms}-like tyrosine kinase-1 receptor (flt-1) and the kinase insert domain-containing receptor (KDR). The recently identified neuropilin-1 enhances binding of VEGF$_{165}$ to KDR and regulates VEGF-induced bioactivity. In the present study, we examined the production and localization of VEGF and its receptors in KD vascular lesions.

Methods

Tissues
All tissues studied were formalin fixed and paraffin embedded (Table 1). In addition to cardiac tissues, we studied lung, kidney, liver, and gall bladder tissues from 2 patients (patients 3 and 5). These tissues demonstrated inflammatory infiltrate in vascular lesions. For controls, cardiac tissues from 5 noncardiac patients and normal lung segments from 6 adults were studied.

Antibodies
Antibodies used in the present study are listed in Table 2. The working dilution of platelet and endothelial cell adhesion molecule-1 (PECAM-1) was 1:20. Other antibodies were diluted 1:100 or 1:200.

Immunohistochemistry
Immunohistochemistry was performed as described previously with some modifications. Single- and double-stain procedures were performed with a labeled streptavidin biotin kit (Dako Japan Co, Ltd) and EnVision Doublestain System (Dako), respectively. Morphometric analysis was performed as described previously.

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fixed paraffin-embedded normal kidney tissues were used as a positive control for VEGF or its receptors.5

**Patients and Serum VEGF**

We studied 71 KD patients (aged 3 months to 10 years) who were treated with intravenous γ-globulin (400 mg · kg⁻¹ · d⁻¹ for 5 days) plus oral aspirin 30 mg · kg⁻¹ · d⁻¹. Measurement of VEGF was performed as reported previously.6 The assay measures the level of VEGF₁₆₅. In general, serum samples were collected before and after therapy, as well as in the convalescent phase (C-reactive protein <0.3). Serum albumin levels were analyzed in the same samples. None of the patients received an albumin infusion. Informed consent for serum studies was obtained from the parents.

**Statistical Analysis**

All data are shown as mean ± SD. The percentage of positive vessels or cells was compared by Mann-Whitney test. Correlation between parameters was tested with Pearson’s correlation coefficient. P<0.05 was considered significant.

**Results**

**Acute Kawasaki Disease Vessels**

Coronary vessels of acute KD had significant upregulation of both VEGF and flt-1 compared with those of controls (P<0.001). VEGF and flt-1 were positive in all 50 coronary vessels studied regardless of vascular sizes or types (Figure 1). In control coronary vessels, VEGF was weakly positive in 33% and flt-1 in 30%.

In nonaneurysmal vessels, VEGF and flt-1 were coexpressed in endothelium and medial smooth muscle cells (SMCs). In aneurysmal arteries, accumulating SMCs in the neointima were positive for VEGF and flt-1. Moreover, VEGF immunostaining was present in the extracellular matrix in VEGF-positive inflammatory infiltrates of aneurysms. The expression and localization of VEGF or flt-1 in other

**TABLE 1. Patient Characteristics of Acute Stage of KD**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age at Death</th>
<th>Ethnicity</th>
<th>Time After Onset</th>
<th>Therapy</th>
<th>Cause of Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1*</td>
<td>3 y</td>
<td>Japanese</td>
<td>7 d</td>
<td>ASA+GG</td>
<td>Congestive heart failure</td>
</tr>
<tr>
<td>2</td>
<td>10 y</td>
<td>White</td>
<td>13 d</td>
<td>ASA+GG</td>
<td>Ruptured CAA</td>
</tr>
<tr>
<td>3</td>
<td>4 y</td>
<td>Japanese</td>
<td>14 d</td>
<td>ASA</td>
<td>Ruptured CAA</td>
</tr>
<tr>
<td>4</td>
<td>11 mo</td>
<td>White</td>
<td>15 d</td>
<td>None</td>
<td>Ruptured CAA</td>
</tr>
<tr>
<td>5</td>
<td>3 mo</td>
<td>Japanese</td>
<td>18 d</td>
<td>ASA+GG</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>6</td>
<td>8 mo</td>
<td>Unknown</td>
<td>3 wk</td>
<td>None</td>
<td>Ruptured CAA</td>
</tr>
<tr>
<td>7</td>
<td>6 mo</td>
<td>White</td>
<td>5 wk</td>
<td>Solu-Cortef</td>
<td>Myocardial infarction</td>
</tr>
</tbody>
</table>

ASA indicates aspirin; CAA, coronary artery aneurysm; and GG, gamma globulin.

*Patient 1 died of third attack of acute KD.

**TABLE 2. Antibodies Used in This Study**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit anti-VEGF*</td>
<td>Santa Cruz Biotechnology Inc</td>
</tr>
<tr>
<td>Rabbit anti-KDR</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Rabbit anti-flt-1</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>Goat anti-neuropilin-1</td>
<td>Santa Cruz</td>
</tr>
<tr>
<td>HAM56 anti-macrophages</td>
<td>Dako Japan Co, Ltd</td>
</tr>
<tr>
<td>CD45RO anti-T cells</td>
<td>Dako</td>
</tr>
<tr>
<td>Mouse anti-smooth muscle cells</td>
<td>Dako</td>
</tr>
<tr>
<td>PECAM-1 anti-endothelial cells</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti-fibrinogen/fibrin</td>
<td>Dako</td>
</tr>
<tr>
<td>Rabbit anti-albumin</td>
<td>Dako</td>
</tr>
</tbody>
</table>

PECAM indicates platelet and endothelial cell adhesion molecule.

Antibodies against VEGF react with VEGF₁₂₁, VEGF₁₆₅, and VEGF₁₈₉.

**Figure 1.** VEGF and flt-1 expression. A, Positive control of VEGF (red) in kidney tissues. B, Abundant VEGF expression in aneurysm of patient 1. C, Medial SMCs and endothelium reactive for VEGF in microvessels of patient 3. Cardiac myocardium is positive for VEGF. D, Pulmonary artery in patient 5. Double staining with anti-VEGF antibody (brown) and anti-HAM56 antibody (red) without nuclear counterstain. VEGF-positive macrophages (black) in perivascular lesion. E, Gall bladder of patient 5. VEGF expression in capillary and venule endothelium. F, Positive control of flt-1 in kidney tissue. Flt-1 is expressed in urinary tubular cells (red) but not in artery (arrow). G and H, Flt-1 is expressed on coronary venous endothelium in patient 1 (G) but not in control patient (H). Cardiac myocardium is positive for flt-1 (H). Bars in A, D, and F=100 μm; B=200 μm; C, E, G, and H=50 μm.
organs (Figure 1) were similar to the findings in the heart. In control lung tissues, VEGF and flt-1 were positive only in 37% and 40% of arteries, respectively.

Coronary vessels of acute KD did not have significant upregulation of KDR or neuropilin-1 compared with those of controls. Expression of KDR was limited to the early stage of acute KD (patient 1) and in 1 of the controls. KDR was positive in the endothelium or medial SMCs of small to medium-sized vessels (30 to 300 μm) at 7 days of illness but negative in the coronary vessels at 2 to 5 weeks of illness (Figure 2). Neuropilin-1 was weakly expressed in the vessels (30 to 330 μm) at 7 to 15 days of illness (Figure 2) but rarely later in the illness. Approximately 60% of control coronary vessels were positive for neuropilin-1.

Infiltrating Cells
VEGF and flt-1 composed 66±14% and 48±16% of infiltrating cells, respectively. KDR was not expressed on infiltrating cells. In aneurysmal lesions, VEGF was found to reside in macrophages (51% of VEGF-positive cells), T cells (20%), and migrating SMCs (9%). Flt-1 was detected on macrophages (66% of flt-1-positive cells) and migrating SMCs (4%) but not on T cells.

Plasma Proteins in Acute Kawasaki Disease Tissues
Acute KD lesions were extensively reactive with anti-fibrinogen/fibrin or anti-albumin antibodies (Figure 3). Staining was prominent in the perivascular lesion associated with edema. By contrast, two thirds of control vessels did not show such staining with these antibodies (P<0.001).

Serum VEGF and Albumin in Acute Kawasaki Disease
Serum VEGF levels were inversely related to serum albumin levels (n=220; r=−0.53, P<0.001) in acute KD (Figure 4). There was no relation between serum VEGF and albumin levels in control sera.

Discussion
The pathology of KD vasculitis is characterized by systemic edema and mononuclear cell infiltrate. Increased microvascular permeability is an important event during the initial process of KD vasculitis. In the present study, enhanced VEGF expression was seen in vessels of many organs in acute KD, which suggests a systemic effect. In aneurysmal arteries, flt-1 was expressed in the accumulating SMCs in the neointima.

KDR signaling appears to be critical for all endothelial responses to VEGF. In acute KD, however, expression of KDR was limited to the early phase of acute KD, 7 days after onset. Expression of neuropilin-1 was also limited to the early stage of acute KD. Neuropilin-1 acts as a coreceptor for VEGF, enhancing its binding to KDR and regulating VEGF-induced microvascular permeability and angiogenesis. Because normal coronary vessels do not express KDR, upregulation of KDR in coronary vessels may suggest VEGF-induced bioactivities in the early stage of acute KD.

The precise mechanisms by which KDR expression was downregulated are unknown. One possibility is that KDR or neuropilin-1 is only upregulated in the initial limited phase of acute KD. Indeed, skin rash or edema of hands and feet is clinically observed shortly after the onset of KD. Elevation of plasma VEGF is already documented at the appearance of skin rash or edema of extremities shortly after KD onset. In fatal cases we studied, endothelial cell injury may have progressed without repair, which would accelerate vascular leakage after the downregulation of VEGF signal receptor. Another possible explanation is the effect of transforming growth factor-β1 or tumor necrosis factor-α, both of which inhibit KDR expression in endothelial cells. Previous studies have documented upregulation of these molecules in acute KD.

Figure 3. A, Positive staining with anti-fibrinogen/fibrin antibody (dark red). KD perivascular lesions are edematous, and extracellular matrix is strongly immunoreactive for anti-fibrinogen/fibrin antibody. B, Control patient. Perivascular region shows much reduced staining compared with that seen in KD. All bars=50 μm.

Figure 4. Correlation of VEGF and albumin levels. Normal values are albumin >3.8 g/dL and VEGF <459 pg/mL.
In conclusion, the present findings suggest that there is systemic production of VEGF and flt-1 in acute KD. KDR expression was limited to the early phase of acute KD. Enhanced VEGF expression in acute KD may result in vascular hyperpermeability and macrophage activation. Low serum albumin may indicate increased in vivo VEGF production in acute KD.

Acknowledgments
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