Increased Production of Vascular Endothelial Growth Factor-D and Lymphangiogenesis in Acute Kawasaki Disease

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Background: Kawasaki disease (KD) is characterized by systemic vasculitis with tissue edema. During the healing process of inflammation, lymphangiogenesis is essential for reducing tissue edema. One potential responsible candidate for the induction of lymphangiogenesis in the healing process of acute KD is vascular endothelial growth factor-D (VEGF-D).

Methods and Results: Sequential changes in serum VEGF-D levels in patients with acute KD (n=47) using an enzyme-linked immunosorbent assay were investigated. Cross-sectional areas of lymphatic vessels and VEGF-D protein expression were evaluated immunohistochemically in cardiac tissues of patients (n=6) who died of KD. Regulation of VEGF-D messenger RNA (mRNA) expression in cultured fibroblasts was assessed using quantitative real-time polymerase chain reaction. Serum VEGF-D levels increased after intravenous immunoglobulin therapy in patients with acute KD (P<0.001). In addition, they were significantly lower in patients with coronary artery lesions (CAL) than in those without CAL (P<0.05). The cross-sectional areas of lymphatic vessels in cardiac tissues were enlarged in patients with acute KD. VEGF-D protein was detected on the endothelium of the enlarged lymphatic vessels. In vitro, tumor necrosis factor-α significantly down-regulated VEGF-D mRNA expression in cultured fibroblasts (P=0.004).

Conclusions: This study indicates that the production of VEGF-D increases and is related to lymphangiogenesis in patients with acute KD. In addition, low VEGF-D production appears to be associated with the development of CAL. (Circ J 2011; 75: 1455–1462)

Key Words: Coronary artery lesions; Kawasaki disease; Lymphangiogenesis; Vascular endothelial growth factor-D

Kawasaki disease (KD) is the most common systemic vasculitis syndrome in children, affecting small and medium-sized arteries—particularly the coronary arteries. Although KD is, in most cases, a self-limiting illness, resolving within a few weeks after fever onset, 15–25% of patients develop coronary artery lesions (CAL) without receiving appropriate therapy. For example, intravenous immunoglobulin therapy (IVIG) has been shown to effectively reduce systemic inflammation and the incidence of CAL. A certain number of patients still develop CAL. They require anti-coagulation therapy, percutaneous coronary intervention, or coronary artery bypass grafting.

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Vascular leakage and tissue edema are key features during the initial phase of KD. Tissue edema causes local hypoxia in the media of the coronary arteries by lowering the oxygen supply from the dietary arteries and plays a role in the development of CAL. Thus, the degree of edema...
serves as an important clinical parameter for predicting the prognosis of patients with KD.10,11  
Lymphatic vessels provide key routes for the drainage of interstitial fluid from tissues. Edema is a cardinal sign of inflammation and a clinically significant feature of inflammatory diseases. It is caused by an excessive amount of leakage from inflamed blood vessels, compared with the capacity of the lymphatic vessels for drainage. Therefore, lymphangiogenesis is essential for reducing tissue edema during the healing process of inflammation.13  
Vascular endothelial growth factor-D (VEGF-D) is a potent lymphangiogenic stimulator that is secreted by fibroblasts, endothelial cells, vascular smooth muscle cells, and activated macrophages.14–18 To stimulate lymphangiogenesis, VEGF-D binds and activates VEGF receptor-3, which is expressed on lymphatic endothelial cells (LEC).14,15 In a mouse tumor model, VEGF-D was reported to stimulate lymphangiogenesis and to promote the metastatic spread of tumor cells via lymphatic vessels.19 Moreover, high circulating VEGF-D levels are correlated with poor prognosis for patients with prostate cancer20 and acquired immunodeficiency syndrome with Kaposi’s sarcoma.21  
However, little is known about the role of VEGF-D in KD. We hypothesized that VEGF-D might stimulate lymphangiogenesis and reduce tissue edema during the healing process of KD. To test our hypothesis, we investigated the production of VEGF-D and its relationship to lymphangiogenesis in patients with acute KD.

Methods

Study Population and Blood Samples  
Patients treated at Chiba University Hospital, Japan, between January 2002 and September 2005 were included in this study. All patients displayed at least 5 major symptoms and were diagnosed as having KD. Patients who did not receive IVIG were excluded from the study. A total of 47 patients (27 boys and 20 girls; age range, 3–95 months; mean age, 37 months) were enrolled in this study. Of these 47 patients, 41 patients were initially treated at Chiba University Hospital. The remaining 6 patients were initially treated at other hospitals and were referred to Chiba University Hospital for additional therapy either because they remained febrile (n=3) after an initial IVIG or because they developed CAL (n=3). Medical records were available for all patients. Parental informed consent was obtained for each child enrolled in this study, which was approved by the Research Ethics Committee of Chiba University Hospital.

Echocardiography  
Two-dimensional echocardiography was performed before and after IVIG as well as at 2, 3, and 4 weeks after the onset of fever. A coronary artery with a diameter of 3 mm or more (4 mm if the patient was over the age of 5 years), as measured using echocardiography, was defined as CAL.22 The presence of CAL was assessed 1 month after the onset of KD.

Measurement of Serum VEGF-D Levels  
Serum VEGF-D levels were measured using a commercially available enzyme-linked immunosorbent assay kit (R&D Systems, Minneapolis, MN, USA), according to the manufacturer’s instructions. Serum samples were collected before IVIG (on days 3–8 of the illness; mean, 5.1 days) and within 2 days after the completion of IVIG (on days 6–11 of the illness; mean, 8.5 days). Serum samples obtained from 16 patients at 1 year after onset were randomly selected. The serum VEGF-D levels in 11 healthy children and 3 patients with non-cyanotic congenital heart disease without heart failure were also studied as control samples. The results were expressed as the mean ± standard error.

Immunohistochemistry  
Formalin-fixed and paraffin-embedded sections of cardiac tissues from 4 fatal cases of acute KD (Patient Numbers 1–4, who died 18, 14, 7, and 18 days after onset, respectively) were analyzed. Cardiac tissues from 2 KD patients who died late after disease onset (Patient Numbers 5 and 6, who died 2 and 24 years after onset, respectively) and 3 fatal cases without cardiac disease (Patient Numbers 7–9) were also analyzed.  
The following 3 antibodies were used: mouse monoclonal anti-human VEGF-D (clone: 78923.11, isotype IgG1; R&D Systems); mouse monoclonal anti-human D2–40 (clone: D2–40, isotype IgG1; Dakocytomation, Carpinteria, CA, USA) for lymphatic vessels; and rabbit polyclonal anti-human Flt-4 (sc-321; Santa Cruz Biotechnology, Santa Cruz, CA, USA) for VEGF receptor-3. After the sections were deparaffinized and rehydrated, immunohistochemistry was performed as described previously.23,24 To identify VEGF-D and VEGF receptor-3, the sections were autoclaved for antigen retrieval. Primary antibodies were applied to the sections overnight at 4°C. The subsequent procedures were performed using the EnVision System (Dakocytomation). Hematoxylin was used as a counterstain. Either non-immune mouse IgG1 or non-immune rabbit IgG was used instead of the respective primary antibodies as a negative control. Cardiomyocytes were used as positive controls for VEGF-D.25

Morphometric Measurement of Lymphatic Vessels  
Morphology of D2–40-positive lymphatic vessels in the sections of cardiac tissues were observed using microscopy and the cross-sectional area of each lymphatic vessel was measured using NIH Image software (National Institutes of Health, Bethesda, MD, USA).

RNA Extraction From Peripheral Blood Mononuclear Cells (PBMC)  
Human PBMC from 4 acute KD patients before and after IVIG were isolated using centrifugation on a Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) density gradient. Total RNA was extracted from the PBMC using ISOGEN (Nippongene, Toyama, Japan), according to the manufacturer’s instructions.

Cell Culture, Tumor Necrosis Factor (TNF)-α Treatment, and RNA Extraction  
Human coronary artery endothelial cells, human coronary artery smooth muscle cells, human neonatal dermis microvascular LEC, and human lung fibroblasts (NLHF) (all from Cambrex Bio Science, Walkersville, MD, USA) were grown in EGM-2MV, SmGM-2, EGM-2MV, and FGM-2 (Cambrex Bio Science), respectively. For the TNF-α treatment, pre-confluent NLHF grown in FGM-2 were maintained in FBM-2 (serum-free basement medium; Cambrex Bio Science) for 18 h. Human recombinant TNF-α (10 ng/ml; R&D Systems) was added, and the cells were incubated for another 24 h. Total RNA was extracted using RNeasy plus (QIAGEN GmbH, Hilden, Germany), according to the manufacturer’s instructions. All assays were performed in duplicate in 3 separate experiments.
Table 1. Clinical Characteristics of Patients and Controls

<table>
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<th>KD</th>
<th>Controls</th>
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<tbody>
<tr>
<td>n</td>
<td>47</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>Age at onset (median)</td>
<td>3–95 months (32 months)</td>
<td>6–134 months (50 months)</td>
<td>NS</td>
</tr>
<tr>
<td>Male/Female ratio</td>
<td>29/18</td>
<td>10/4</td>
<td>NS</td>
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KD, Kawasaki disease; NS, statistically not significant.

Table 2. Clinical Characteristics of KD Patients With CAL and Without CAL

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<th>KD without CAL</th>
<th>KD with CAL</th>
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<td>n</td>
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<td>10</td>
<td></td>
</tr>
<tr>
<td>Age at onset (median)</td>
<td>3–94 months (27 months)</td>
<td>3–95 months (58 months)</td>
<td>0.012</td>
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<tr>
<td>Male/Female ratio</td>
<td>23/14</td>
<td>6/4</td>
<td>NS</td>
</tr>
<tr>
<td>IVIG (total 2 g/kg)</td>
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<td></td>
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<tr>
<td>Single infusion</td>
<td>0</td>
<td>1</td>
<td>NS</td>
</tr>
<tr>
<td>2-day infusion</td>
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<td>7</td>
<td></td>
</tr>
<tr>
<td>4-day infusion</td>
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<td>4-day infusion</td>
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KD, Kawasaki disease; CAL, coronary artery lesion; NS, statistically not significant; IVIG, intravenous immunoglobulin therapy.

Quantitative Real-Time Polymerase Chain Reaction (PCR)
Total RNA was reverse-transcribed to complementary DNA (cDNA) using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random hexamers (GE Healthcare, Buckinghamshire, UK). The PCR primers and probe for glyceraldehyde phosphate dehydrogenase (GAPDH) were purchased from Applied Biosystems (Assays-on-Demand; Gene Expression Products, assay no. Hs99999905; Foster City, CA, USA). The primers and probe for VEGF-D were designed based on sequences from GenBank. The primer sequences were as follows: forward primer, 5'-TTGCTGGAACAGAAGAC-CACCTCT-3'; reverse primer, 5'-TCGCAACGATCTTCGTTCAAA-3'. Quantitative real-time PCR was conducted using the ABI 7300 sequence detector system (Applied Biosystems). The expression of target cDNA relative to GAPDH was calculated using a comparative Tc method described in User Bulletin 2, provided by the manufacturer (Applied Biosystems), and was determined for each sample. All assays were performed in duplicate. Data are shown as the relative expression after normalization for the GAPDH signals.

Statistical Analysis
The difference in the number of patients between groups was analyzed by the chi-square test for independence and Fisher’s exact possibility test. Pair-wise comparisons of serum VEGF-D levels were performed using the Mann–Whitney U-test. The Wilcoxon signed-rank test was used to compare the serum VEGF-D levels before and after IVIG. The Mann–Whitney U-test was also used to compare the serum VEGF-D levels between patients with and those without CAL and to analyze the effect of TNF-α on the VEGF-D mRNA expression. As the measured cross-sectional areas of the lymphatic vessels were normally distributed after a logarithmic transformation, logarithmically transformed values (normal logarithm) were used. These values were compared using the Kruskal-Wallis test, followed by pair-wise comparisons using the Mann–Whitney U-test. In addition, a hierarchical cluster analysis was performed using R software (R Development Core Team, Vienna, Austria) to evaluate similarities in the cross-sectional areas of the lymphatic vessels in each patient. A P-value was adjusted using Holm’s method. A P-value <0.05 was considered statistically significant.

Results
Clinical Characteristics of Patients and Controls
The baseline characteristics of the patients and controls are detailed in Table 1. No significant differences between patients with KD and controls were found. As shown in Table 2, 10 patients in the KD group had CAL. The male/female ratio was not significantly different between KD patients with CAL and those without CAL, but age was higher (P=0.012) in those with CAL. All of them received IVIG (2 g/kg) and the regimen, single infusion, 2-day infusion or 4-day infusion, was not significantly different between KD patients with CAL and those without CAL.
Figure 2. Morphometric measurements of lymphatic vessels. (A) Representative pictures of lymphatic vessels in a patient with acute Kawasaki disease (KD; Left panel, 14 days after onset) and a patient in the late phase of the disease (Right panel, 2 years after onset). Bars=50 μm. (B) Cross-sectional areas of lymphatic vessels in acute KD patients (Patient numbers 1–4), patients in the late phase of the disease (Patient numbers 5 and 6), and those without cardiac diseases (Patient numbers 7–9). The Y-axis indicates the logarithmically transformed cross-sectional areas of the lymphatic vessels. The data are expressed as a box-and-whiskers graph. The line at the middle is the median. The box indicates the interquartile range. The whiskers extend down to the lowest value and up to the highest. The cross-sectional areas of the lymphatic vessels were significantly different among these participants, as analyzed using a Kruskal–Wallis test (P<0.001). (C) Cluster dendrogram for the log-transformed cross-sectional areas of the lymphatic vessels. Patient numbers are listed along the X-axis in an order convenient for showing the cluster structure. The Y-axis measures height (inter-cluster distance). Patient 1 had the largest cross-sectional area of lymphatic vessels among these participants. The participants were divided into 2 groups: Patient numbers 1–4 and Patient numbers 5–9.
Serum VEGF-D Levels
The serum VEGF-D levels of the 47 patients and 14 controls are shown in Figure 1A. The serum VEGF-D levels of the KD patients at 1 year after disease onset and those of the controls were 356±27 pg/ml (range, 164–561 pg/ml) and 388±34 pg/ml (range, 166–557 pg/ml), respectively. The serum VEGF-D levels before IVIG (397±28 pg/ml, range 37–1,157 pg/ml) were not significantly different from those of the controls and the KD patients at 1 year after onset. After IVIG, the serum VEGF-D levels increased (727±56 pg/ml; range, 245–2,022 pg/ml; P<0.001) and were significantly higher than those of the controls (P<0.001) and the KD patients at 1 year after onset (P<0.001).

In addition, we studied whether serum VEGF-D levels were associated with the development of CAL (Figure 1B). After IVIG, serum VEGF-D levels significantly increased in both the patients with CAL (299±32 pg/ml to 488±63 pg/ml, P=0.011) and those without CAL (422±33 pg/ml to 788±65 pg/ml, P<0.001). However, serum levels of VEGF-D were significantly lower in patients with CAL than those without CAL before IVIG (P=0.039) as well as after IVIG (P=0.011).

In our study, no gender-based difference in serum VEGF-D levels was seen (data not shown).
Morphometric Measurement of Lymphatic Vessels
The cross-sectional areas of the lymphatic vessels were significantly larger in patients with acute KD than in those who died well after onset and those without cardiac disease (Figures 2A, B). First, a significant difference in the cross-sectional area of the lymphatic vessels was observed among these participants using the Kruskal–Wallis test (P<0.001). Pair-wise comparisons using the Mann–Whitney U-test revealed that each group (Patient Numbers 2–4; Patient Numbers 5 and 6; and Patient Numbers 6–9) had significant intergroup differences and no intragroup differences in the cross-sectional areas of their lymphatic vessels (Table 3). In addition, these participants were divided into 2 groups, Patient Numbers 1–4 and Patient Numbers 5–9, using a hierarchical cluster analysis (Figure 2C). These findings were consistent with a distinction between patients with acute KD and the other patients.

Immunohistochemical Detection of VEGF-D and Its Receptor
VEGF-D was detected on cardiomyocytes as a positive control and vascular smooth muscle cells in the cardiac tissues of patients with acute KD (Figure 3A). On the endothelium of enlarged lymphatic vessels, VEGF-D and its receptor, VEGF-receptor 3, were detected (Figures 3B–D). Furthermore, VEGF-D and VEGF-receptor 3 were detected on vascular endothelial cells, fibroblasts and inflammatory infiltrates around the enlarged lymphatic vessels in the cardiac tissues of patients with acute KD (Figures 3C, D).

Expression and Regulation of VEGF-D mRNA
VEGF-D mRNA expression in PBMC from acute KD patients before and after IVIG was extremely low, compared with the other types of cells that were examined. Among the other types of cells, VEGF-D mRNA expression was the most abundantly expressed in NHLF (Figure 4).

As shown in Figure 5, in NHLFs, TNF-α dramatically down-regulated VEGF-D mRNA expression (P=0.004).

Discussion
This study indicates that the production of VEGF-D increases in KD patients after IVIG and is related to lymphangiogenesis during the healing process of KD.

Production of VEGF-D Increases in Patients With Acute KD
In our study, the serum VEGF-D levels nearly doubled after IVIG. We measured the VEGF-D concentration in immunoglobulin and confirmed that the therapeutic amount of immunoglobulin did not affect serum VEGF-D levels (data not shown). These results indicate that the production of VEGF-D increases after IVIG in patients with KD.

To identify where VEGF-D is produced in patients with KD, VEGF-D protein expression in cardiac tissue was evaluated immunohistochemically and VEGF-D mRNA expression in PBMC was evaluated using quantitative real-time PCR. Our study showed that VEGF-D protein was detected in various types of cardiac tissue cells, such as cardiomyocytes, vascular smooth muscle cells, lymphatic and vascular endothelial cells, fibroblasts and inflammatory infiltrates. However, VEGF-D mRNA expression was extremely low in PBMC. These results are compatible with previous studies in which VEGF-D mRNA was found to be most abundant in the heart and was not detected in circulating leukocytes. The low production of VEGF-D mRNA in PBMC was also reported previously in a gene expression profile analysis of acute KD.
patients. Kaushal et al reported that circulating levels of VEGF-D were closely related to VEGF-D mRNA and protein expression levels in prostate cancer specimens. These findings support the idea that VEGF-D is produced in some cardiac tissue cells and that its production increases after IVIG in patients with acute KD.

Role of VEGF-D in Acute KD

VEGF-D, which binds and activates VEGF receptor-3, stimulates lymphangiogenesis. Previous studies reported that serum or plasma VEGF-D levels were increased under pathological conditions that promoted lymphangiogenesis, such as cancer metastasis via lymphatic vessels, and hereditary lymphedema. Since lymphangiogenesis is essential for reducing tissue edema during the inflammation healing process, the increased serum VEGF-D levels in acute KD patients after IVIG suggest the involvement of lymphangiogenesis during the healing process in KD.

Lymphatic vessels are thought to be maintained in a collapsed state under physiological conditions, and their enlargement is indicative of pathological conditions requiring the drainage of interstitial fluid. For example, Nagy et al reported that lymphatic capillaries enlarged progressively in parallel with LEC proliferation, and Baluk et al reported that the enlargement of lymphatic vessels resulted from the proliferation of endothelial cells combined with distention from accumulating lymphatic. In our study, a microscopic assessment of cardiac tissue showed that the lymphatic vessels were enlarged in patients with acute KD, compared with those in the controls and KD patients in a late phase of the disease. In addition, immunohistochemical staining showed that VEGF-D and its receptor, VEGF receptor-3, were detected on lymphatic vessels, vascular endothelial cells, fibroblasts and inflammatory infiltrates around the enlarged lymphatic vessels in patients with acute KD. Previous studies reported VEGF-D may play an important role in cancer metastasis via lymphatic vessels through paracrine and autocrine mechanisms. In cases of organ transplantation, lymphatic vessels contribute to drainage of lymphatic fluid and rejection infiltrates. VEGF receptor-3 is expressed in LEC, dendritic cells, and macrophages in corneal transplant and cardiac allograft. It has recently been shown that VEGF-D and VEGF receptor-3 are critical in antigen clearance and inflammation resolution through mobilization of inflammatory cells from inflamed tissue to the draining lymph nodes in models of skin inflammation and arthritis. In these cases, the lymphangiogenic growth factors are secreted from infiltrated macrophages, local inflamed tissue and draining lymph nodes. These findings suggest that VEGF-D is one of the factors that contribute not only to lymphangiogenesis, but also to mobilization of inflammatory infiltrates to lymphatic systems through paracrine and autocrine mechanisms in patients with acute KD.

VEGF-D and Prognosis of KD Patients

If lymphangiogenesis is essential for the healing process in KD, serum VEGF-D levels might predict the prognosis for KD patients. Therefore, we compared serum VEGF-D levels between patients with and those without CAL. Interestingly, serum VEGF-D levels were significantly lower in patients with CAL than in those without CAL both before and after IVIG. These results suggest that production of VEGF-D is low in patients with CAL, leading to less active lymphangiogenesis and drainage of inflammatory infiltrates. We speculate that, in these patients, prolonged inflammation and tissue edema, which might result in an insufficient oxygen supply from the dietary arteries to the coronary arteries, might cause the development of CAL.

At present, the reason why serum VEGF-D levels were lower in the KD patients with CAL than in patients without CAL remains uncertain. In our study, TNF-α dramatically down-regulated VEGF-D mRNA expression in cultured fibroblasts. This finding suggests that TNF-α influences lower serum VEGF-D levels in KD patients with CAL. Previous studies reported that circulating levels of TNF-α were elevated in patients with acute KD, especially in those with CAL. In a mouse model of KD, Hui-Yuen et al reported high levels of TNF-α in affected vascular tissue during the development of CAL. More importantly, the blockade of TNF-α activity is expected to be a novel therapy for patients with KD who fail to respond to IVIG. The precise mechanism by which VEGF-D mRNA expression is down-regulated by TNF-α is unknown. Orlandini et al found that VEGF-D mRNA expression was down-regulated by the activation of the Wnt signaling pathway. Because the Wnt signaling pathway is augmented by TNF-α, TNF-α might indirectly down-regulate VEGF-D mRNA expression.

Study Limitations

We were unable to perform sequential morphometric measurements of the lymphatic vessels or to compare measurements between patients with and those without CAL because the number of autopsy cases of acute KD was limited. We also were unable to quantify the mRNA or protein levels of VEGF-D in tissues because freshly resected tissues were not available.

Conclusions

The present study indicates that VEGF-D is produced in some cardiac tissue cells around enlarged lymphatic vessels and that its production increases after IVIG in patients with acute KD. In addition, low VEGF-D production appears to be associated with the development of CAL. VEGF-D might play a role in the healing process in KD, such as lymphangiogenesis. However, further research is required to investigate whether VEGF-D plays an active role in the healing process in KD or simply represents an epiphenomenon.

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Disclosure

Conflict of interest: There are no financial associations that might pose a conflict of interest in connection with this study.

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