Suppressed Production of Soluble Fms-Like Tyrosine Kinase-1 Contributes to Myocardial Remodeling and Heart Failure

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Abstract—Soluble fms-like tyrosine kinase-1 (sFlt-1), an endogenous inhibitor of vascular endothelial growth factor and placental growth factor, is involved in the pathogenesis of cardiovascular disease. However, the significance of sFlt-1 in heart failure has not been fully elucidated. We found that sFlt-1 is decreased in renal failure and serves as a key molecule in atherosclerosis. In this study, we aimed to investigate the role of the decreased sFlt-1 production in heart failure, using sFlt-1 knockout mice. sFlt-1 knockout mice and wild-type mice were subjected to transverse aortic constriction and evaluated after 7 days. The sFlt-1 knockout mice had significantly higher mortality (52% versus 15%; P=0.0002) attributable to heart failure and showed greater cardiac hypertrophy (heart weight to body weight ratio, 8.95±0.45 mg/g in sFlt-1 knockout mice versus 6.60±0.32 mg/g in wild-type mice; P<0.0001) and cardiac dysfunction, which was accompanied by a significant increase in macrophage infiltration and cardiac fibrosis, than wild-type mice after transverse aortic constriction. An anti-placental growth factor–neutralizing antibody prevented pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. Moreover, monocyte chemoattractant protein-1 inhibition with neutralizing antibody ameliorated monocyte chemoattractant protein-1 expression in pressure-overloaded heart.

Key Words: chronic kidney disease ■ heart failure ■ hypertrophy/remodeling ■ monocyte chemoattractant protein-1 ■ placental growth factor

Heart failure is a multifactorial syndrome that results from myocardial ischemia, chronic volume overload, or chronic pressure overload, and patients with heart failure face a substantial risk of hospitalization and mortality worldwide. Pressure overload–induced heart failure because of hypertension or aortic valve stenosis is characterized by initial compensated cardiac hypertrophy and subsequent decompensated heart failure.1

Fms-like tyrosine kinase 1 (Flt-1) is a member of the vascular endothelial growth factor (VEGF) receptor family and binds to VEGF-A and placental growth factor (PLGF) and stimulates angiogenesis and vascular permeability with recruiting and activating macrophages. A soluble isoform of Flt-1 (sFlt-1), produced by alternative splicing of full-length Flt-1 mRNA, lacks the transmembrane and intracellular domain of Flt-1 and regulates the availability of free VEGF and PLGF in peripheral circulation.2 Recently, it has been shown that sFlt-1 is implicated in the pathogenesis of cardiovascular disease.3–10

In a series of previous studies,3,4,11 we demonstrated that sFlt-1 production is decreased in patients with chronic kidney disease (CKD) and in an experimental model of renal failure1 and that decreased sFlt-1 production correlates with the development of atherosclerosis and cardiovascular events.3,4 Furthermore, previous reports showed that the gene delivery of sFlt-1 suppresses atherosclerosis development in an animal model,5 and the increase in sFlt-1 levels induced by atorvastatin treatment is associated with improvement of ventricular

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function in patients with acute coronary syndrome. Therefore, it seems that insufficient sFlt-1 production is associated with adverse cardiovascular outcomes.

On the contrary, several previous reports have shown that upregulation of sFlt-1 contributes to the development of heart failure, with antiangiogenesis activity by binding to VEGF,\textsuperscript{7,8} In clinical settings, plasma levels of sFlt-1 are not only directly correlated with the severity of heart failure but also strongly associated with poor outcomes in patients with heart failure.\textsuperscript{9,10} These observations provide a plausible interpretation that increased sFlt-1 production aggravates heart failure with adverse cardiac remodeling by sequestering angiogenic factors. However, little is known about how decreased sFlt-1 production is involved in the development of cardiac hypertrophy and heart failure. We hypothesized that not only elevated but also suppressed production of sFlt-1 is involved in the pathogenesis of developing and worsening heart failure.

The aim of our study was to examine the significance of the contribution of sFlt-1 to the development of cardiac hypertrophy and heart failure. Therefore, we created sFlt-1\textsuperscript{−/−} mice and performed transverse aortic constriction (TAC) to elucidate the precise role of sFlt-1 in heart failure.

## Methods

An expanded Methods section is provided in the online-only Data Supplement.

### Animals

We used 10- to 12-week-old sFlt-1 knockout mice from a C57BL/6 background and their wild-type (WT) littermates. Generation of sFlt-1 knockout mice was described previously.\textsuperscript{11} Briefly, we created constitutive sFlt-1 knockout mice in which intron 13 of the sFlt-1 gene was deleted and exon 13 was directly connected to exon 14, thus preventing alternative splicing at intron 13. Full-length Flt-1 was preserved in these mice. Although sFlt-1 mRNA was completely abrogated in sFlt-1\textsuperscript{−/−} mice, sFlt-1-like immunoreactivity was detected in plasma at about half the levels in WT mice. It was assumed that other splicing variants of Flt-1 or other isoforms generated by shedding of the extracellular domain of Flt-1 caused this condition.\textsuperscript{12–14} TAC was performed as described previously.\textsuperscript{15} Briefly, mice were intubated and anesthetized using 1% to 2% isoflurane. Respiration was controlled by mechanical ventilation (SN-480–7; Shinano Manufacturing Co, Ltd, Tokyo, Japan). After the chest cavity was opened, the transverse aorta was ligated by tying a 6-0 nylon suture against a 27-gauge needle. The needle was immediately removed. Sham animals underwent open chest surgery without constriction. TAC and sham operations were performed by the same operator. To investigate the effect of sFlt-1 replacement, we administered recombinant human sFlt-1 protein\textsuperscript{1} intraperitoneally at a dose of 15 ng per gram of body weight every day for 2 weeks, beginning when the mice were 10 weeks old. After a week, the mice were subjected to the TAC procedure and euthanized 7 days after TAC.

To examine the role of PLGF/Flt signaling during pressure overload, we administered an anti-PLGF neutralizing antibody (αPLGF) after TAC in both sFlt-1\textsuperscript{−/−} and WT mice. The αPLGF and control immunoglobulin G were produced by ThromboGenics N.V. (Leuven, Belgium). Mice were injected with αPLGF (50 mg/kg) or control immunoglobulin G (50 mg/kg) intraperitoneally 3 times a week after TAC. Furthermore, we investigated the effect of monocyste chemotactrant protein-1 (MCP-1) inhibition on the development of cardiac fibrosis and hypertrophy after TAC. We administered 20 μg of a goat anti-mouse MCP-1 antibody (AB479-NA; R&D Systems, Minneapolis, MN) or goat immunoglobulin G (AB-108C; R&D Systems) intraperitoneally for 7 days after TAC for both sFlt-1\textsuperscript{−/−} and WT mice. Mice were euthanized and analyzed 7 days after the procedure. We excluded the expired animals. All animal experimental protocols were conducted in accordance with the Guidelines for Animal Experiments at Nara Medical University and were approved by The Animal Research and Ethics Committee of Nara Medical University, Nara, Japan, and conformed to National Institutes of Health guidelines in effect at that time. Adequate anesthetic and analgesics were used to reduce pain in the mice during and after surgery.

### Echocardiography

Echocardiographic studies (SSA-770A; Toshiba, Tokyo, Japan) were performed before and 3 and 7 days after TAC. All mice were anesthetized with 1.5% isoflurane. The left ventricular end-systolic dimensions, left ventricular end-diastolic dimensions, left ventricular posterior wall thickness dimensions, and interventricular wall thickness dimensions were measured. The LV ejection fraction and fractional shortening were calculated using M-mode tracings.

### Blood Pressure and Hemodynamic Measurements

Blood pressure and heart rate were measured in conscious mice using a noninvasive tail-cuff system (BP-98A; Softron Co, Tokyo, Japan) after sham or TAC operation. Invasive hemodynamics was evaluated by using a Millar catheter (SPR-671; Millar Instruments, Houston, TX). A Millar catheter was inserted into the left ventricle under anesthesia to measure systolic pressure and end-diastolic pressure after sham or TAC operation.

### mRNA Analysis and Western Blot

mRNA levels were detected by reverse transcription quantitative real-time polymerase chain reaction of frozen left ventricular tissue. Protein content was determined by Western blot (see the online-only Data Supplement for more detail).

### Histology

Mice were euthanized, and hearts, lungs, and kidneys were harvested at day 7 after TAC. Hearts were harvested, fixed with buffered 4% formalin, and embedded in paraffin. Then, 5-μm-thick sections were obtained and stained by Masson’s trichrome for the detection of myocardial interstitial fibrosis.

### Immunohistochemistry and Immunofluorescence

Details for immunohistological analysis are provided in the online-only Data Supplement.

### ELISA

Blood samples were obtained from mice by eye bleeding after sham or TAC operation. Serum levels of mouse sFlt-1 were measured with commercial sandwich ELISA (R&D systems, Minneapolis, MN).

### Statistical Analysis

Differences between the 2 groups were determined by Student’s t test, Mann–Whitney U test, or χ\textsuperscript{2} test. Multiple groups were performed with 1-way ANOVA, followed by the Tukey post-test or the Bonferroni test. Serum sFlt-1 levels after sham or TAC operation were analyzed with 2-way factorial ANOVA and the Bonferroni post hoc test. Data are presented as mean±SEM. Survival analysis was performed using Kaplan–Meier curves with log-rank test for comparison between the groups. P<0.05 was considered statistically significant. All statistical analyses were performed with GraphPad Prism, version 5 (GraphPad Software, Inc, La Jolla, CA).

## Results

### Decrease in sFlt-1 Production Exacerbates Cardiac Hypertrophy, Remodeling, and Heart Failure in Response to Pressure Overload

To elucidate the effect of decreased sFlt-1 production on heart failure, we examined sFlt-1\textsuperscript{−/−} mice and their littermates. The mice were subjected to TAC and evaluated after 7 days. There were no significant differences either in the pressure gradient,
systolic pressure, or end-diastolic pressure of the left ventricle after TAC between WT and sFlt-1−/− mice (Figure S1 in the online-only Data Supplement) or in the heart rate or blood pressure between WT and sFlt-1−/− mice after sham or TAC operation (Table S1). Survival curve showed that almost half of sFlt-1−/− mice died within 7 days after TAC, although 85% of WT mice survived (Figure 1A). The major cause of death in sFlt-1−/− mice was heart failure, as indicated by higher lung weight/body weight (LW/BW). Both the heart weight/BW ratio and the LW/BW ratio were significantly increased in sFlt-1−/− compared with WT mice after 7 days of TAC (Figure 1B and 1C), which indicated that sFlt-1−/− mice are susceptible to heart failure by pressure overload.

Echocardiography 7 days after TAC demonstrated that left ventricle wall thickness (interventricular wall thickness dimensions and posterior wall thickness dimensions) was significantly increased and the ejection fraction was significantly decreased in the sFlt-1−/− compared with the WT mice (Figure 1D and Table). The number of macrophages in sFlt-1−/− mice was markedly elevated compared with that in WT mice 7 days after TAC (Figure 2A and 2B). Double staining for CD68 and CD206 revealed that M1 macrophage (CD206−CD68+) accumulation was significantly increased in sFlt-1−/− compared with WT mice 7 days after TAC (Figure 2E and 2F), suggesting that Flt-1 signaling enhanced the proinflammatory response in sFlt-1−/− mice by pressure overload. The increase in myocardial interstitial fibrosis by Masson’s trichrome staining was significantly greater in sFlt-1−/− versus WT mice (Figure 2C and 2D). Immunohistochemical analysis showed that capillary density in the myocardium, as determined by the number of vessels to cardiomyocyte ratio, was significantly increased in sFlt-1−/− mice compared with WT mice after TAC, accompanied by greater growth of cardiomyocytes (Figure S2). Although circulating levels of both VEGF and PLGF in sFlt-1−/− mice were not different from those in WT mice (data not shown), the circulating sFlt-1 level in sFlt-1−/− mice after sham

Figure 1. The decrease in soluble fms-like tyrosine kinase-1 (sFlt-1) production enhanced cardiac hypertrophy and heart failure, contributing to high mortality after transverse aortic constriction (TAC).

A, Kaplan–Meier survival curves for mice subjected to TAC operation; n=40 for wild-type (WT) and n=60 for sFlt-1−/− (knockout [KO]). P=0.0002. B and C, The heart weight/body weight (HW/BW) ratio (B) and the lung weight/body weight (LW/BW) ratio (C) in WT and sFlt-1−/− (KO) mice, who survived 7 days after TAC. WT and sFlt-1−/− mice treated with recombinant human sFlt-1 (rhsFlt-1) 7 days after TAC, WT mice (n=3) and KO mice (n=5) for TAC-operated mice treated with rhsFlt-1 (TAC+rhsFlt-1). D, Representative transthoracic M-mode echocardiograms for WT and sFlt-1−/− mice (KO) 7 days after sham operation or TAC; WT and sFlt-1−/− (KO)+rhsFlt-1 7 days after TAC. ***P<0.001 vs sham, †††P<0.001 vs WT TAC, ‡‡‡P<0.01 vs KO TAC. Data are mean±SEM.
operation was significantly lower by about half compared with that in WT mice (Figure S3). Time-dependent increases of circulating sFlt-1 levels were not observed in both WT and sFlt-1−/− mice after TAC (Figure S3).

**Recombinant sFlt-1 Protein Administration**

To determine whether the decreased sFlt-1 production contributes to cardiac hypertrophy, remodeling, and heart failure by pressure overload, we investigated the effect of sFlt-1 replacement. After intraperitoneal administration of recombinant sFlt-1 protein into the sFlt-1−/− mice, the heart weight/BW ratio and cardiac hypertrophy by echocardiography remained at a level similar to that observed in WT mice after TAC (Figure 1B and 1D). Echocardiographic analysis also showed that administration of recombinant sFlt-1 protein rescued left ventricular hypertrophy and cardiac dysfunction in sFlt-1−/− mice after TAC (Figure 1D and Table). In addition, sFlt-1 replacement significantly reduced the LW/BW ratio in sFlt-1−/− mice but not in WT mice after TAC (Figure 1C). Moreover, we also observed that administration of recombinant sFlt-1 significantly suppressed the increase of

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<th>TAC (n=7)</th>
<th>TAC+sFlt-1 (n=3)</th>
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<th>TAC (n=8)</th>
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<td>0.78±0.02‡</td>
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<td>PWd, mm</td>
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<td>79.16±0.96‡</td>
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Echocardiographic analysis revealed that left ventricular wall thickness (IVSd and PWd) was significantly increased and ejection fraction was significantly decreased in sFlt-1−/− vs WT mice 7 days after TAC. Recombinant sFlt-1 protein administration prevented the deterioration of cardiac hypertrophy and left ventricular systolic dysfunction after pressure overload in sFlt1−/− mice. EF indicates ejection fraction; IVSd, interventricular wall thickness dimensions; LVDd, left-ventricular end-diastolic dimensions; PWd, posterior wall thickness dimensions; sFlt-1, soluble fms-like tyrosine kinase-1; TAC, transverse aortic constriction; and WT, wild-type.

*P<0.05 vs corresponding sham group.
†P<0.05 vs WT TAC.
‡P<0.05 vs sFlt-1−/− TAC. Data are mean±SEM.
macrophage infiltration in sFlt-1−/− mice after TAC (Figure 2A and 2B). In addition, remarkably, the increase of cardiac fibrosis in sFlt-1−/− mice after TAC was attenuated by administration of recombinant sFlt-1 (Figure 2C and 2D). These findings suggest that sFlt-1 replacement reduced the progression of cardiac remodeling and heart failure in sFlt-1−/− mice after TAC.

Anti-PLGF–Neutralizing Antibody Rescues Pressure Overload–Induced Cardiac Hypertrophy and Heart Failure

sFlt-1 serves as a decoy receptor for both VEGF-A and PLGF. PLGF/Flt-1 signaling acts not only as an angiogenic factor, but also as a proinflammatory cytokine by mobilizing macrophages. Therefore, we investigated whether PLGF/Flt-1 signaling influences the development of cardiac remodeling and heart failure during pressure overload under the condition of the suppressed sFlt-1 production by administration of αPLGF into sFlt-1−/− and WT mice. αPLGF suppressed the increase of the heart weight/BW ratio to baseline levels in sFlt-1−/− mice after TAC (Figure 3A). Additionally, administration of αPLGF significantly reduced the LW/BW ratio in sFlt-1−/− mice after TAC (Figure 3B). Echocardiographic analysis showed that αPLGF prevented pressure overload–induced cardiac hypertrophy and dysfunction in sFlt-1−/− mice after TAC (Table S2). Histological findings revealed that αPLGF attenuated pressure overload–induced macrophage infiltration and cardiac fibrosis in sFlt-1−/− mice (Figure 3C–3F). On the basis of these results, we speculated that PLGF enhances the proinflammatory response by mobilizing macrophages and contributes to promoting cardiac remodeling in sFlt-1−/− mice during pressure overload.

Figure 3. Placental growth factor (PLGF) neutralization prevented pressure overload–induced cardiac hypertrophy, macrophage infiltration, and fibrosis in sFlt-1−/− mice. A and B, The heart weight/body weight (HW/BW) ratio (A) and the lung weight/body weight ratio (B) in wild-type (WT) and sFlt-1−/− (knockout [KO]) mice treated with an anti-PLGF neutralizing antibody (αPLGF) or control immunoglobulin G (IgG) 7 days after transverse aortic constriction (TAC) or sham operation. C, Representative immunostaining of macrophages in cardiac sections with CD68. D, Representative images of cardiac fibrosis with Masson’s trichrome staining. E, Quantification of CD68-positive cells per 1 mm². F, Quantification of cardiac fibrotic area. n=6 for sham-operated mice treated with control IgG (sham+IgG); n=4 for sham-operated mice treated with αPLGF (sham+αPLGF); n=7 to 9 for TAC-operated mice treated with control IgG (TAC+IgG); n=7 for TAC-operated mice treated with αPLGF (TAC+αPLGF). ***P<0.001 vs sham-treated with IgG and αPLGF. †††P<0.001 vs WT TAC+IgG, ‡‡‡P<0.001 vs KO TAC+IgG. Data are mean±SEM. Scale bar, 40 μm. Magnification, x400. sFlt-1 indicates soluble fms-like tyrosine kinase-1.
Upregulation of MCP-1 Expression Contributes to the Progression of Cardiac Hypertrophy and Heart Failure

PLGF is known to act as a chemotactic agent for monocytes and increases the expression of cytokines, including MCP-1, in normal monocytes. Previous reports showed that various inflammatory cytokines participate in the progression of maladaptive cardiac remodeling during pressure overload. Notably, MCP-1 reportedly contributes to adverse cardiac remodeling in response to pressure overload and ischemic stimuli in animal models. Therefore, we investigated whether MCP-1 was expressed in the hypertrophied hearts of the sFlt-1−/− mice. In our study, the mRNA expression of MCP-1 was significantly upregulated in sFlt-1−/−mice 3 days after TAC (Figure 4A). MCP-1 protein production was upregulated in sFlt-1−/− compared with WT mice 7 days after TAC, as determined by Western blotting (Figure 4B). Furthermore, MCP-1 was strongly induced not only in macrophages but also in endothelial cells, interstitial cells, and cardiomyocytes in sFlt-1−/− mice, even though MCP-1 protein was detected only in the infiltrating cells in WT mice (Figure 4C; Figure S4).

The anti-MCP-1–neutralizing antibody prevented pressure overload–induced cardiac hypertrophy and dysfunction in sFlt-1−/− mice, but not in WT mice (Figure 5A; Table S3). The anti-MCP-1–neutralizing antibody also inhibited the infiltration of macrophages (Figure 5B and 5D) and development of cardiac fibrosis (Figure 5C and 5E) in sFlt-1−/− mice during pressure overload.

Discussion

The major findings of this study are as follows: (1) the decrease in sFlt-1 production was a precipitating factor for progression to cardiac hypertrophy, fibrosis, and heart failure with pressure overload; (2) PLGF worked as a ligand for Flt-1–mediated cardiac remodeling in sFlt-1−/− mice after TAC; (3) MCP-1, as a downstream signaling molecule of the PLGF/Flt-1 signaling, played a role in infiltration of M1 macrophages...
and subsequent cardiac remodeling and heart failure in sFlt-1−/− mice after TAC.

Previously, we demonstrated that sFlt-1 production is decreased in CKD patients and in an experimental model of renal failure and that PLGF production increases in patients with CKD in accordance with its severity. Considering our previous clinical findings that the higher levels of PLGF and the lower levels of sFlt-1 are significantly associated with a greater risk of cardiovascular events, including heart failure, we speculated that the suppressed production of sFlt-1 and activation of PLGF/Flt-1 signaling play a key role in the development of heart failure. In this study, we demonstrated that a decrease in sFlt-1 production exacerbates pressure overload–induced cardiac hypertrophy and heart failure with mobilizing activated macrophages and excessive fibrosis.

Real-time PCR analysis demonstrated that PLGF and Flt-1 gene expressions were upregulated in the hearts of sFlt-1−/− compared with WT mice; however, there was no difference in VEGF mRNA between sFlt-1−/− and WT mice (Figure S5). Therefore, we focused on PLGF/Flt-1 signaling. To investigate whether PLGF/Flt-1 signaling works in sFlt-1−/− mice after TAC, we administered anti-PLGF antibody in sFlt-1−/− and WT mice after TAC. Interestingly, the anti-PLGF antibody apparently inhibited the progression of cardiac remodeling and the development of heart failure in sFlt-1−/− mice after TAC. Thus, these findings indicate that PLGF works as an important factor for the development of heart failure in sFlt-1−/− mice during pressure overload.

PLGF has been shown to promote macrophage mobilization and act as a proinflammatory cytokine in various pathological disorders. In addition, PLGF activates monocytes and increases the expression of cytokines, including MCP-1, in normal monocytes. In our previous study, MCP-1 mRNA was upregulated in peritoneal macrophages in sFlt-1−/− mice. Furthermore, the present study revealed that macrophages
were skewed into the CD68⁺CD206⁻ inflammatory phenotype, and that MCP-1 expression was significantly increased in the pressure-overloaded hearts of sFlt-1⁻/⁻ compared with the WT mice. On the basis of these findings, it is likely that MCP-1 acts downstream of PLGF/Flt-1 signaling. To test this idea, we examined the effect of MCP-1 inhibition. MCP-1 inhibition by MCP-1 neutralizing antibody almost completely inhibited the infiltration of macrophages in the pressure-overloaded heart, as well as cardiac hypertrophy and heart failure in the present study, suggesting that MCP-1 working downstream of the PLGF/Flt-1 signaling partially plays a role in the proinflammatory and cardiac maladaptive responses of Flt-1⁻/⁻ mice after TAC. In addition, on the basis of previously reported findings that the cardiac expression of MCP-1 may be involved in the progression of maladaptive cardiac remodeling and decompensation,26-28 the present study suggests that the upregulation of MCP-1 in hypertrophied hearts of sFlt-1⁻/⁻ mice may be one of the reasons for maladaptive cardiac remodeling.

Thus, the present study revealed that decreased sFlt-1 production exacerbates pressure overload–induced cardiac remodeling and heart failure, which seems a maladaptive response to pressure overload. Possible mechanisms for adverse outcomes in decreased sFlt-1 production are (1) activation of MCP-1 by relative activation of the PLGF/Flt-1 signaling because of a decrease in a decoy isoform, as mentioned above; and (2) disruption of cellular function because of reduction of sFlt-1, which was recently reported in renal podocytes and pericytes from other tissues.39 sFlt-1 binds to glycosphingolipid on the surface of podocytes to control the cells’ function by reorganizing their cytoskeleton. Decrease in sFlt-1 consequently leads to massive proteinuria and renal dysfunction. Similar cell function of sFlt-1 itself is observed in various vascular pericytes from many tissues.

In contrast, previous studies have reported the adverse effect of elevated levels of sFlt-1. Overexpression of sFlt-1 in hypertrophied myocardium reportedly contributes to the development of heart failure by inhibiting angiogenesis and impairing adaptive cardiac hypertrophy.7 Patten et al demonstrated that exogenous administration of adenovirus expressing sFlt-1 causes diastolic dysfunction in WT mice, accompanied by a decrease in vascular density.80 Di Marco et al also revealed that exogenous administration of sFlt-1 causes heart failure by reducing heart capillary density and myocardial blood flow, with interstitial fibrosis and mitochondrial damages in rats.8 Importantly, angiogenic factors are considered to have an important role in the process of adaptive cardiac response to pressure overload.31-33 These results suggest that higher than normal levels of sFlt-1 may result in adverse effects on cardiac function by binding and sequestering angiogenic factors, leading to impaired angiogenesis. In contrast, the number of vessels to cardiomyocyte ratio was increased in sFlt-1⁻/⁻ mice in response to pressure overload, accompanied by greater growth of cardiomyocytes in the present study (Figure S2). These earlier works together with our findings raise the possibility that both higher than normal levels and lower than normal levels of sFlt-1, in other words, nonphysiological levels of sFlt-1, may lead to dysregulation of angiogenesis and inflammatory process. In the present study, the decreased levels of sFlt-1 may participate in the aggravation of pressure overload–induced cardiac hypertrophy and fibrosis by enhancing an inflammatory response, although the possibility that the increment of capillary density may be insufficient for hypertrophied ventricle could not be excluded. In clinical settings, elevated plasma levels of sFlt-1 are correlated with the severity of heart failure.9,10 In addition, administration of excessive sFlt-1 reportedly results in endothelial dysfunction and proteinuria.34,35 On the basis of these findings, we concluded that both extremely elevated and suppressed production of sFlt-1 have an adverse effect in vivo, indicating that the normalization of endogenous sFlt-1 would be appropriate.

In addition, the early development of heart failure in sFlt-1⁻/⁻ mice, as indicated by higher LW/BW ratio, is less likely to be associated with pressure overload–induced cardiac remodeling and dysfunction. These results raise the possibility that other causes such as vascular hyperpermeability in the lungs could contribute to the early development of heart failure in sFlt-1⁻/⁻ mice. It has been reported that overexpression of VEGF in the lungs induces vascular permeability, contributing to pulmonary edema.36,37 Furthermore, PLGF signaling reportedly synergizes with VEGF signaling to induce angiogenesis7 and vascular permeability,17 and antibodies against PLGF reduce plasma extravasation in mice.17 Considering our finding that the anti-PLGF–neutralizing antibody reduced the LW/BW ratio in sFlt-1⁻/⁻ mice after TAC, the synergistic activation of PLGF and VEGF under the condition of the suppressed sFlt-1 production may participate in inducing early death by heart failure or hyperpermeability in the lungs. Further study will be necessary to elucidate the mechanism for the development of heart failure in the downregulation of sFlt-1.

Patients with CKD are well known to be at high risk of developing heart failure.18 Furthermore, we demonstrated that sFlt-1 production is decreased in patients with CKD and in an experimental model of renal failure.14 These findings seem to be contradicting other studies.8,39 Actually, we proved that the expression levels of sFlt-1 mRNA are downregulated in the renal tissues of both CKD model mice and CKD patients.14 Furthermore, we found that intravenous injection of heparin increases the plasma levels of sFlt-1. Given that sFlt-1 has heparin-binding domains, through which sFlt-1 binds extracellular matrix of cellular surface, heparan sulfate, it is plausible that exogenously administered heparin probably releases the stored sFlt-1 from the surface of the endothelial cells into peripheral blood. Plasma sFlt-1 levels after heparin injection, in other words, postheparin sFlt-1 levels, are positively correlated with estimated glomerular filtration rate in CKD patients,4 although preheparin plasma levels of sFlt-1 were increased with the progression of renal failure in agreement with the previous reports.38,39 These findings indicate that postheparin sFlt-1 levels can be a surrogate marker for the total amount of sFlt-1 production.4 We found that postheparin sFlt-1 levels are reduced by approximately half in dialysis patients compared with healthy subjects.4 On the basis of these findings, we propose that the suppressed production of sFlt-1 exhibits one of the characteristics of CKD. These results raise the possibility that the suppressed production of sFlt-1 may play some role in the progression of CKD–associated heart failure.
It is well known that higher sFlt-1 levels are associated with elevated blood pressure in patients with preeclampsia. However, the clinical significance of sFlt-1 in blood pressure remains unclear. One potential limitation of our study is the measuring method of blood pressure in mice. We measured blood pressure by tail cuff method at a specific time point, which is unlikely to represent blood pressure variability as determined by measuring 24-hour blood pressure. Thus, unrecognized group differences in blood pressure may affect cardiac outcomes in the present study.

Another possible limitation of our study is the use of sFlt-1−/− mice. Circulating sFlt-1 in sFlt-1−/− mice was detected, although the levels were about half of those in WT mice (Figure S3). This was assumed to be because of the presence of other splicing variants of Flt-1 or other isoforms generated by shedding of the extracellular domain of Flt-1. Although circulating sFlt-1 levels were not increased in both WT mice and sFlt-1−/− mice after TAC (Figure S3), it is unclear whether reserved sFlt-1, which was not circulating but stored on endothelial cell surfaces, was generated by pressure overload. Furthermore, it is also unknown when and how other soluble isoforms of Flt-1 are produced and act in vivo. Further study is needed to elucidate the functional mechanisms of sFlt-1.

In conclusion, the present study demonstrates that decreased production of sFlt-1 augments TAC-induced macrophage infiltration in the ventricle, cardiac hypertrophy, fibrosis, and heart failure through activation of MCP-1 signaling.

Perspectives

This study demonstrates that suppressed production of sFlt-1 contributes to adverse cardiac remodeling and the development of heart failure with pressure overload, which could be caused by relative activation of PLGF/Flt-1 signaling. Upregulation of MCP-1 expression in pressure-overloaded heart, as a downstream signaling molecule of the PLGF/Flt-1 signaling, is also associated with maladaptive response to pressure overload. Our findings highlight the importance of maintaining physiological level of endogenous sFlt-1.

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References


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Title: SUPPRESSED PRODUCTION OF SOLUBLE FMS-LIKE TYROSINE KINASE-1 CONTRIBUTES TO MYOCARDIAL REMODELING AND HEART FAILURE

Ayako Seno¹, Yukiji Takeda¹, Masaru Matsui¹, Aya Okuda¹, Tomoya Nakano¹, Yasuki Nakada¹, Takuya Kumazawa¹, Hitoshi Nakagawa¹, Taku Nishida¹, Kenji Onoue¹, Satoshi Somekawa¹, Makoto Watanabe¹, Hiroyuki Kawata¹, Rika Kawakami¹, Hiroyuki Okura¹, Shiro Uemura¹*, and Yoshihiko Saito¹,²

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Short Title: sFlt-1 and Heart Failure

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SUPPLEMENTAL MATERIAL

Supplemental Methods

Immunohistology and Immunofluorescence
Cardiac tissue was fixed and frozen in OCT-embedding compound (Tissue Tek; Sakura Finetek, Tokyo, Japan) at -80°C. Then, 10-µm-thick sections were obtained and fixed with cold acetone. The following antibodies were used for immunohistology: rat anti-CD68 (dilution 1/10,000) (ab53444; Abcam, Cambridge, MA, USA) and mouse anti-MCP-1(dilution 1/2,000) (2D8; Nobus Biologicals, Littleton, CO, USA). Images were obtained by a fluorescent microscope (BZ-X700; KEYENCE, Osaka, Japan). The optional software (BZ-analysis; KEYENCE) was used for the analysis.

For immunofluorescent staining, 10-µm cryosections were fixed with cold acetone and blocked with 10% normal goat serum (Sigma-Aldrich, St. Louis, MO, USA). Sections were incubated for two hours at room temperature with primary antibodies. For analysis of microvessels and myocytes, rat anti-CD31 (dilution 1/100; BD Biosciences: 550274 and Wheat germ agglutinin Alexa Fluor 488 (WGA, dilution 1/1000; Invitrogen: W11261) were used. For analysis of macrophages, rat anti-CD68 and goat anti-CD206 (dilution 1/200) (AF2535; R&D Systems) were used. Secondary antibodies were as follows: Alexa Fluor 594 donkey anti-rat and Cy2 donkey anti-goat.

Macrophages, fibroblasts, endothelial cells, and myocytes were double stained with MCP-1 and cell-specific surface antigens in order to investigate localization of MCP-1 protein in the pressure-overloaded heart. Primary antibodies were as follows: mouse anti-MCP1 (dilution 1/500) (2D8; Nobus Biologicals), rat anti-CD68, rat anti-fibroblasts (dilution 1/400) (ER-TR7; Acris Antibodies GmbH, San Diego, CA, USA), rat anti-CD31 (dilution 1/100) (550274; BD Biosciences, Franklin Lakes, NJ, USA), and rabbit anti-Cardiac Troponin I (dilution 1/100) (ab47003; Abcam) followed by staining with secondary antibody: Alexa Fluor 488 anti-rat, Alexa Fluor 488 anti-rabbit, and Alexa Fluor 647 anti-mouse. Images of immunofluorescent staining were obtained by confocal microscopy (FLUOVIEF FV1000l; Olympus, Tokyo, Japan). The analyses for cardiomyocyte areas and the number of vessels-to-cardiomyocyte ratio were performed using Image J 1.46 software (https://imagej.nih.gov/ij).

Real-time Polymerase Chain Reaction
Cardiac mRNA was extracted from the left ventricle using Trizol reagent (Life Technologies, CA, USA). Expression levels of sFlt-1 and Flt-1 mRNA were measured as described previously.1 Reverse transcription was performed using the QuantiTect Reverse Transcription Kit (One Step Real-time PCR Systems; Life Technologies, Grand Island, NY, USA). Levels of gene expression were quantified by real-time polymerase chain reaction using Taqman Gene
Expression Assays (One Step Real-time PCR Systems; Life Technologies).

**Western Blotting**
The cardiac tissues were homogenized with lysis buffer (pH 7.6, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 10 mM DTBA, 8M urea). The total 10-µg sample was loaded on 16% gel (TEFCO) and transferred to membrane. The membrane was blocked with Blocking One (Nacalai Tesque, Inc., Kyoto, Japan) for 20 minutes, and incubated overnight at 4° C with mouse anti-MCP-1 antibody (dilution 1/1,000) (2D8; Nobus Biologicals). Anti-mouse IgG, HRP-linked antibody (dilution 1/5,000) (#7076; Cell Signaling Technology, Danvers, MA, USA) was used as a secondary antibody for one hour at room temperature. The signals were detected by a SuperSignal West Dura chemiluminescent substrate (Fisher Scientific, Pittsburgh, PA, USA). The blots were also probed with a monoclonal GAPDH antibody (dilution 1/100,000) (Sigma-Aldrich) as a control.

**References**
**Supplemental Table**

**Table S1. Blood pressure measurement.**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>sFlt-1&lt;sup&gt;−/−&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham (n = 4)</td>
<td>TAC (n = 3)</td>
</tr>
<tr>
<td>HR, /min</td>
<td>603.57±35.23</td>
<td>566.2±71.7</td>
</tr>
<tr>
<td>SBP, mmHg</td>
<td>103.5±3.53</td>
<td>106.87±2.89</td>
</tr>
<tr>
<td>MBP, mmHg</td>
<td>72.57±2.61</td>
<td>72.67±3.04</td>
</tr>
<tr>
<td>DBP, mmHg</td>
<td>57.4±3.27</td>
<td>55.57±3.66</td>
</tr>
</tbody>
</table>

There were no significant differences in heart rate or blood pressure between wild-type (WT) and sFlt-1<sup>−/−</sup> mice after sham or TAC operation. DBP indicates diastolic blood pressure; HR, heart rate; MBP, mean blood pressure; SBP, systolic blood pressure.
Table S2. Echocardiographic analysis for mice treated with control IgG or anti-PLGF neutralizing antibody (αPLGF)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>WT</th>
<th>TAC</th>
<th>sFlt-1−/−</th>
<th>TAC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sham</td>
<td>TAC</td>
<td>Sham</td>
<td>TAC</td>
</tr>
<tr>
<td>IgG (n=6)</td>
<td>aPLGF (n=4)</td>
<td>IgG (n=9)</td>
<td>aPLGF (n=8)</td>
<td>IgG (n=6)</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>0.60±0.02</td>
<td>0.63±0.01</td>
<td>0.80±0.01*</td>
<td>0.70±0.01†</td>
</tr>
<tr>
<td>PWd, mm</td>
<td>0.61±0.02</td>
<td>0.63±0.01</td>
<td>0.81±0.01*</td>
<td>0.73±0.02‡</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>2.67±0.11</td>
<td>2.72±0.10</td>
<td>2.52±0.13</td>
<td>2.91±0.15</td>
</tr>
<tr>
<td>EF, %</td>
<td>77.97±1.46</td>
<td>78.03±1.04</td>
<td>77.01±1.01</td>
<td>78.01±2.80†</td>
</tr>
</tbody>
</table>

Treatment with αPLGF rescued cardiac hypertrophy and left ventricular systolic dysfunction after pressure overload in sFlt-1−/− mice. IgG indicates immunoglobulin G; IVSd, interventricular wall thickness dimensions; LVDd, left-ventricular end-diastolic dimensions; PLGF, placental growth factor; PWd, posterior wall thickness dimensions; sFlt-1, soluble Flt-1; TAC, transverse aortic constriction; WT, wild-type.

*P < 0.05 vs. corresponding sham group; †P < 0.05 vs. WT (TAC + IgG); ‡P < 0.05 vs. sFlt-1−/− (TAC + IgG). Data are mean ± SEM.
Table S3. Echocardiographic analysis for mice treated with control IgG or MCP-1 neutralizing antibody (MCP-1Ab)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sham (n = 7)</th>
<th>TAC (n = 8)</th>
<th>Sham (n = 7)</th>
<th>TAC (n = 8)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgG</td>
<td>MCP-1Ab</td>
<td>IgG</td>
<td>MCP-1Ab</td>
</tr>
<tr>
<td>IVSd, mm</td>
<td>0.61±0.02</td>
<td>0.58±0.02</td>
<td>0.78±0.01*</td>
<td>0.76±0.02†</td>
</tr>
<tr>
<td>PWd, mm</td>
<td>0.61±0.02</td>
<td>0.63±0.01</td>
<td>0.81±0.01*</td>
<td>0.76±0.02†</td>
</tr>
<tr>
<td>LVDd, mm</td>
<td>2.65±0.04</td>
<td>2.48±0.03</td>
<td>2.93±0.16</td>
<td>2.84±0.14</td>
</tr>
<tr>
<td>EF, %</td>
<td>78.18±0.93</td>
<td>77.63±1.29</td>
<td>75.67±2.23</td>
<td>77.23±2.21†</td>
</tr>
</tbody>
</table>

Treatment with MCP-1Ab prevented pressure-overloaded cardiac hypertrophy and dysfunction in sFlt-1−/− mice. IgG indicates immunoglobulin G; IVSd, interventricular wall thickness dimensions; LVDd, left-ventricular end-diastolic dimensions; MCP-1Ab, monocyte chemoattractant protein-1 antibody; PWd, posterior wall thickness dimensions; sFlt-1, soluble Flt-1; TAC, transverse aortic constriction; WT, wild-type.

*P < 0.05 vs. corresponding sham group; †P < 0.05 vs. WT (TAC + IgG); ‡P < 0.05, ‡P < 0.05 vs. sFlt-1−/− (TAC + IgG). Data are mean ± SEM.
Invasive hemodynamic measurement demonstrated that there was no difference in the pressure gradient, systolic pressure, and end-diastolic pressure of left ventricle after transverse aortic constriction (TAC) between wild-type (WT) and sFlt-1⁻/⁻ mice (KO). A,
representative recordings of left ventricular pressure in sham- or TAC-operated mice. B, left ventricular systolic pressure. C, left ventricular pressure gradient. D, left ventricular end-diastolic pressure.

***$P < 0.001$ vs. sham. Data are mean ±SEM.
Figure S2

A, Representative double staining by immunofluorescence of cardiac sections with wheat germ agglutinin (green) and CD31 (red), and DAPI (blue) in wild-type (WT) and sFlt-1−/− mice (KO) seven days after sham or TAC.

Cardiomyocyte areas and the number of vessels to cardiomyocyte in the myocardium were increased in sFlt-1−/− mice in response to TAC.
transverse aortic constriction (TAC) operation. Scale bar, 100 μm. Magnification, × 200. B, Quantification of the number of vessels to cardiomyocyte ratio. C, Quantification of cardiac myocyte cross-sectional area. n = 4 for sham-operated mice (sham), n = 5-6 for TAC-operated mice (TAC). **P<0.01. ***P<0.001 vs sham, †P<0.05, †††P < 0.001 vs. WT TAC. Data are mean ±SEM.
Circulating sFlt-1 levels were measured by ELISA three and seven days after TAC or sham operation in both WT and sFlt-1⁻/⁻ mice. *P<0.05 vs WT, 2-way ANOVA, Bonferroni posttest. P<0.0001 between WT and KO groups by 2-way ANOVA. n = 6-7 per group.
Figure S4.

Representative double staining by immunofluorescence of cardiac sections with MCP-1 (red) and CD68, fibroblast, CD31, troponin I (green) in wild-type (WT) and sFlt-1\(^{-/}\) mice (KO)
seven days after sham or transverse aortic constriction (TAC) operation. Monocyte chemoattractant protein-1 (MCP-1) was mainly expressed in macrophages, but was expressed in endothelial cells, interstitial cells, and cardiomyocytes in sFlt-1−/− mice (KO) during pressure overload. Scale bar, 40 μm. Magnification, × 400.
Cardiac mRNA expression of sFlt-1, Flt-1, placental growth factor (PLGF), and vascular endothelial growth factor (VEGF) in wild-type (WT) mice and sFlt-1−/− mice (KO). n = 8 for WT, n = 7 for sFlt-1−/− mice (KO).

*P < 0.05, **P < 0.01 ***P < 0.001 vs. WT.