Full Title **R3**

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 (VEGF) and placental growth factor (PLGF), 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 seven days. The sFlt-1-knockout mice had significantly higher mortality (52 vs. 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 vs. 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-PLGF neutralizing antibody prevented pressure overload–induced cardiac hypertrophy, fibrosis, and cardiac dysfunction. Moreover, monocyte chemoattractant protein-1 (MCP-1) expression was significantly increased in the hypertrophied hearts of sFlt-1-knockout mice compared to that in wild-type mice. MCP-1 inhibition with neutralizing antibody ameliorated maladaptive cardiac remodeling in sFlt-1-knockout mice after transverse aortic constriction. In conclusion, decreased sFlt-1 production plays a key role in the aggravation of cardiac hypertrophy and heart failure through upregulation of MCP-1 expression in pressure-overloaded heart.

Keywords: heart failure; placental growth factor; monocyte chemoattractant protein-1; chronic kidney disease; hypertrophy/remodeling

Introduction

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 due to 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 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 failure,[3] 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 are associated with improvement of ventricular function in patients with acute coronary syndrome.[6] Therefore, it seems that insufficient sFlt-1 production is associated with adverse cardiovascular outcomes.

On the other hand, several previous reports have shown that upregulation of sFlt-1 contributes to the development of heart failure with anti-angiogenesis activity by binding to VEGF.[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.[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-$1^{-/-}$ 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 WT littermates. Generation of sFlt-1 knockout mice was described previously.[4] Briefly, we created constitutive sFlt-1 knockout mice in which intron 13 of the *sFlt-1* gene was deleted, and exon13 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$^{-/-}$ 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.[12-14] TAC was performed as described previously.[15] Briefly, mice were intubated and anesthetized using 1-2% 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 (rhsFlt-1) protein[3] intraperitoneally at a dose of 15 ng per gram of body weight every day for two weeks, beginning when the mice were 10 weeks old. After a week the mice were subjected to the TAC procedure and sacrificed seven days after TAC.

To examine the role of PLGF/Flt-1 signaling during pressure overload, we administered an anti-PLGF neutralizing antibody (αPLGF) after TAC in both sFlt-1$^{-/-}$ and WT mice. The αPLGF and control immunoglobulin G (IgG) were produced by ThromboGenics N.V. (Leuven, Belgium). Mice were injected with αPLGF (50 mg/kg) or control IgG (50 mg/kg) intraperitoneally three times a week after TAC. Furthermore, we investigated the effect of monocyte chemoattractant 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, USA) or goat IgG (AB-108C; R&D Systems) intraperitoneally for seven days after TAC for both sFlt-1$^{-/-}$ and WT mice. Mice were sacrificed and analyzed seven 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 NIH 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 three and seven days after TAC. All mice were anesthetized with 1.5% isoflurane. The left ventricular end-systolic dimensions (LVESD), left ventricular end-diastolic dimensions (LVEDD), left ventricular posterior wall thickness dimensions (LVPWd), and interventricular wall thickness dimensions (IVSd) were measured. The LV ejection fraction (EF) and fractional shortening (FS) were calculated using M-mode tracings.

Blood Pressure and Hemodynamic Measurements

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

Histology

Mice were sacrificed 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.

Immunohistology 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 kits (R&D systems, Minneapolis, MN).

Statistical Analysis

Differences between the two groups were determined by Student's *t*-test, Mann–Whitney U test, or χ^2 test. Multiple groups were performed with one-way ANOVA, followed by the Tukey posttest or the Bonferroni test. Serum sFlt-1 levels after sham or TAC operation were analyzed with two-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, USA).

Results

A 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-$1^{-/-}$ mice and their littermates. The mice were subjected to TAC and evaluated after seven days. There were no significant differences either in the pressure gradient, systolic pressure, or in end-diastolic pressure of the left ventricle after TAC between WT and sFlt-$1^{-/-}$ mice (Supplemental Figure S1), or in heart rate or blood pressure between WT and sFlt-$1^{-/-}$ mice after sham or TAC operation (Supplemental Table S1). Survival curve showed that almost half of sFlt-$1^{-/-}$ mice died within seven 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/body weight (HW/BW) ratio and the LW/BW ratio were significantly increased in sFlt-$1^{-/-}$ compared with WT mice after seven days of TAC (Figure 1B and 1C), which indicated sFlt-$1^{-/-}$ mice are susceptible to heart failure by pressure overload.

Echocardiography seven days after TAC demonstrated that left ventricle wall thickness (IVSd and PWd) was significantly increased and the ejection fraction was significantly decreased in sFlt-$1^{-/-}$ compared with those in WT mice (Figure 1D, Table 1).

The number of macrophages in sFlt-$1^{-/-}$ mice was markedly elevated compared with WT mice seven 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 seven days after TAC (Figure 2E and 2F), suggesting that Flt-1 signaling enhanced the pro-inflammatory 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). Immunohistological 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 (Supplemental 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 operation was significantly lower by about half compared to that in WT mice (Supplemental Figure S3). Time-dependent increases of circulating sFlt-1 levels were not observed in both WT and sFlt-1$^{-/-}$ mice after TAC (Supplemental 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 HW/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, Table 1). 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 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 pro-inflammatory cytokine by mobilizing macrophages.[11,16-19] 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 HW/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 (Supplemental 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 pro-inflammatory response by mobilizing macrophages and contributes to promoting cardiac remodeling in sFlt-1$^{-/-}$ mice during pressure overload.

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.[20,21] Previous reports showed that various inflammatory cytokines participate in the progression of maladaptive cardiac remodeling during pressure overload.[22] Notably, MCP-1 reportedly contributes to adverse cardiac remodeling in response to pressure overload and ischemic stimuli in animal models.[23-25] Therefore we investigated whether MCP-1 was expressed in the hypertrophied hearts of the sFlt1$^{-/-}$ mice. In our study, the mRNA expression of MCP-1 was significantly upregulated in sFlt-1$^{-/-}$ mice three days after TAC (Figure 4A). MCP-1 protein production was upregulated in sFlt-1$^{-/-}$ compared to that in WT mice seven 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 also cardiomyocytes in sFlt-1$^{-/-}$ mice, even though MCP-1 protein was detected only in the infiltrating cells in WT mice (Figure 4C, Supplemental 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 and Supplemental 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 an experimental model of renal failure,[3] and that PLGF production increases in patients with CKD in accordance with its severity.[4] 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,[4,11] we speculated that the suppressed production of sFlt-1 and activation of PLGF/Flt-1 signaling play a key role of 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 (Supplemental 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 pro-inflammatory cytokine in various pathological disorders.[16-19] In addition, PLGF activates monocytes and increases the expression of cytokines, including MCP-1 in normal monocytes.[20,21] In our previous study, MCP-1 mRNA was upregulated in peritoneal macrophages in sFlt-1$^{-/-}$ mice.[4] 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 that in 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 MCP-1 working downstream of the PLGF/Flt-1 signaling partially plays a role in the pro-inflammatory 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 due to a decrease in a decoy isoform, as mentioned above; and (2) disruption of cellular function due to reduction of sFlt-1, which was recently reported in renal podocytes and pericytes from other tissues.[29] 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.[30] 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 (Supplemental 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, non-physiological 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 angiogenesis[7] 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.[38] Furthermore, we demonstrated that sFlt-1 production is decreased in patients with CKD and in an experimental model of renal failure.[3-4] 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.[3-4] 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, post-heparin sFlt-1 levels, are positively correlated with estimated glomerular filtration rate (eGFR) in CKD patients,[4] although pre-heparin plasma levels of sFlt-1 were increased with the progression of renal failure in agreement with the previous reports.[4,8,39] These findings indicate that post-heparin sFlt-1 levels can be a surrogate marker for the total amount of sFlt-1 production.[4] We found that post-heparin 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.[35,40] Although the physiological function of sFlt-1 is

probably different depending on the presence or absence of pregnancy, previous studies showed that administration of exogenous sFlt-1 in a rat model causes high blood pressure in the absence of pregnancy.[8,35] 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 (Supplemental Figure S3). This was assumed to be due to 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 (Supplemental 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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Novelty and Significance

What Is New?

- Suppressed production of sFlt-1 exacerbates pressure overload-induced cardiac remodeling and heart failure.
- PLGF works as a ligand for Flt-1–mediated cardiac remodeling in sFlt-$1^{-/-}$ mice through upregulation of MCP-1 expression in pressure-overloaded heart.

What is Relevant?

- This study demonstrates the significance of suppressed sFlt-1 production in the pathogenesis of pressure overload-induced cardiac hypertrophy and heart failure.

Summary

- Suppressed sFlt-1 production contributes to the aggravation of cardiac remodeling and heart failure through upregulation of MCP-1 expression in pressure-overloaded heart.

Figure Legend

Figure 1. The decrease in 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 WT and n = 60 for sFlt-$1^{-/-}$ (KO). $^{*}P = 0.0002$. **B-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), who survived seven days after TAC. WT and sFlt-$1^{-/-}$ treated with recombinant human sFlt-1 (rhsFlt-1) seven days after TAC or sham operation. Sham-operated mice (n = 10 per group); WT mice (n = 17), and KO mice (n = 23) for TAC-operated mice. 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) seven days after sham operation or TAC; WT and sFlt-$1^{-/-}$ (KO) + rhsFlt-1 seven days after TAC.

$^{***}P < 0.001$ vs. sham, $^{\dagger\dagger\dagger}P < 0.001$ vs. WT TAC, $^{\ddagger\ddagger}P < 0.01$ vs. KO TAC. Data are mean ± SEM.

Figure 2. The decrease in sFlt-1 production induced macrophage infiltration and fibrosis in the pressure-overloaded heart. **A**, Representative immunostaining of macrophages in cardiac sections with CD68 for WT and sFlt-$1^{-/-}$ (KO) seven days after sham operation or TAC; WT and sFlt-$1^{-/-}$ (KO) + rhsFlt-1 seven days after TAC. **B**, Quantification of CD68-positive cells per $1mm^2$. **C**, Representative images of cardiac fibrosis with Masson's trichrome staining. **D**, Quantification of cardiac fibrotic area. n = 4 for sham-operated mice (sham); n = 7–8 for TAC-operated mice; n = 3 for WT (TAC + rhsFlt-1); n = 5 for sFlt$1^{-/-}$ (KO) (TAC + rhsFlt-1). Scale bar, 40 μm. Magnification, × 400. **E**, Double staining of the left ventricle for $CD68^{+}$ (macrophages in red) and $CD206^{+}$ (M2 marker in green) cells. Merged images ($CD206^{+}CD68^{+}$) in yellow show M2 macrophages; nuclei stained with DAPI (blue). Scale bar, 100 μm. Magnification, × 200. **F**, Quantification revealed infiltration of M1 macrophages ($CD206^{-}$ $CD68^{+}$) was significantly increased in sFlt-$1^{-/-}$ (KO) compared to that in WT mice seven days after TAC procedure. n = 4 for sham-operated mice (sham), n = 8–11 for TAC-operated mice (TAC). $^{**}P < 0.01$,$^{***}P < 0.001$ vs. sham, $^{\dagger\dagger\dagger}P < 0.001$ vs. WT TAC, $^{\ddagger\ddagger}P < 0.01$, $^{\ddagger\ddagger\ddagger}P < 0.001$ vs. KO TAC.

Figure3. PLGF neutralization prevented pressure overload-induced cardiac hypertrophy, macrophage infiltration and fibrosis in sFlt-$1^{-/-}$ mice.

A-B, The heart weight/body weight (HW/BW) ratio (A) and the lung weight/body weight ratio (B) in WT and sFlt-$1^{-/-}$ (KO) mice treated with an anti-PLGF neutralizing antibody

(αPLGF) or control immunoglobulin G (IgG) seven days after 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 1mm^2. **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 = 7–9 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, × 400.

Figure4. Cardiac expression of MCP-1 was significantly increased in sFlt-1$^{-/-}$ compared with that in WT mice after TAC operation. **A**, Cardiac mRNA expression of MCP-1 in WT and sFlt-1$^{-/-}$ (KO) mice three days after TAC operation. mRNA expression was normalized to 18S, represented as fold change to WT sham. n = 7 for sham-operated mice (sham) and n = 6 for TAC-operated mice (TAC). Scale bar, 40 μm. Magnification, × 400. **B**, Representative immunoblots and quantitative analysis for MCP-1 in cardiac tissue of WT and sFlt-1$^{-/-}$ (KO) mice seven days after sham or TAC operation. Results were normalized to GAPDH. n = 3 per group. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. sham, $^{†}P < 0.05$ vs. WT TAC. **C**, Representative immunohistostaining for MCP-1 in cardiac sections seven days after TAC.

Figure5. MCP-1 inhibition ameliorated pressure overload–induced cardiac hypertrophy, macrophage infiltration, and fibrosis in sFlt-1$^{-/-}$ mice. **A**, The heart weight/body weight (HW/BW) ratio in WT and sFlt-1$^{-/-}$ (KO) mice treated with an anti MCP-1 neutralizing antibody (MCP-1Ab) or control IgG seven days after TAC or sham operation. **B**, Representative immunostaining of macrophages in cardiac sections with CD68. **C**, Representative images of cardiac fibrosis with Masson's trichrome staining. **D**, Quantification of CD68 positive cells per 1 mm^2. **E**, Quantification of cardiac fibrotic area. n = 4 for sham-operated mice treated with control IgG (sham + IgG) or MCP-1Ab (sham + MCP-1Ab); n = 8 for TAC-operated mice treated with control IgG (TAC + IgG); n = 8–10 for TAC-operated mice treated with MCP-1Ab (TAC + MCP-1Ab). $^{***}P < 0.001$ vs. sham-treated with IgG and MCP-1Ab. $^{†}P < 0.05$, $^{†††}P < 0.001$ vs. WT TAC + IgG, $^{‡‡}P < 0.01$, $^{‡‡‡}P < 0.001$ vs. KO TAC + IgG. Scale bar, 40 μm. Magnification, × 400.

Table 1. Echocardiographic analysis for WT and sFlt-1$^{-/-}$ mice after TAC

Parameter	WT			sFlt-1$^{-/-}$		
	Sham (n = 5)	TAC (n = 7)	TAC+sFlt-1 (n = 3)	Sham (n = 5)	TAC (n = 8)	TAC+sFlt-1 (n = 5)
IVSd, mm	0.61±0.02	0.79±0.01*	0.75±0.04*	0.61±0.02	0.97±0.03*†	0.78±0.02*‡
PWd, mm	0.62±0.02	0.79±0.01*	0.74±0.06	0.63±0.01	1.07±0.04*†	0.78±0.03*‡
LVDd, mm	2.48±0.18	2.80±0.11	2.60±0.43	2.65±0.20	2.83±0.20	2.79±0.23
EF, %	77.44±1.50	76.81±1.65	76.07±0.71	76.08±1.60	51.50±4.81*†	79.16±0.96‡

Echocardiographic analysis revealed that left ventricular wall thickness (IVSd and PWd) was significantly increased and ejection fraction was significantly decreased in sFlt-1$^{-/-}$ versus WT mice seven days after TAC. Recombinant sFlt-1 protein administration prevented the deterioration of cardiac hypertrophy and left ventricular systolic dysfunction following 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 Flt-1; TAC, transverse aortic constriction; WT, wild-type.

$^{*}P < 0.05$ vs. corresponding sham group; $^{\dagger}P < 0.05$ vs. WT TAC; $^{\ddagger}P < 0.05$ vs. sFlt-1$^{-/-}$ TAC. Data are mean ± SEM.