# ORIGINAL ARTICLE

# Upregulation of soluble vascular endothelial growth factor receptor type 1 by endogenous prostacyclin inhibitor coupling factor 6 in vascular endothelial cells: a role of acidosis-induced c-Src activation

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Vascular endothelial growth factor (VEGF) is a well-known promoter of angiogenesis, but its receptor VEGFR-1 and a soluble short form of VEGFR-1 (sFlt-1) play a negative role in the VEGF signal pathway by trapping VEGF. We recently showed that endogenous prostacyclin inhibitor coupling factor 6 (CF6) forces the clockwise rotation of $F_1$ motor of plasma membrane adenosine triphosphate synthase and induces intracellular acidosis and c-Src activation. We investigated the role of CF6 in regulation of sFlt-1, and its mechanism in human umbilical vein endothelial cells. The ratio of sFlt-1 to glyceraldehyde 3-phosphate dehydrogenase mRNA was increased at 24 h by $1.59 \pm 0.29$-fold by $10^{-7}$ M CF6 ($P < 0.05$), concomitantly with the increases in intercellular adhesion molecule-1 and lectin-like oxidized low-density lipoprotein receptor-1 and no change in VEGF-A. When the dose of CF6 was increased to $10^{-6}$ M, no further increase in sFlt-1 mRNA was observed. The release of sFlt-1 protein was increased by $1.72 \pm 0.24$-fold ($P < 0.05$) at 48 h after exposure to CF6 at $10^{-7}$ M, and it was blocked by pretreatment with anti-CF6 antibody. The immunoreactive bands for sFlt-1 and VEGFR-1 were both increased by CF6 to similar degrees. Pretreatment with PP1, an inhibitor of c-Src, and $10^{-5}$ M efrapeptin, an inhibitor of $F_1$ motor, inhibited CF6-induced increases in expression and release of sFlt-1 ($P < 0.05$). In mice overexpressing CF6, the plasma level of sFlt-1 was increased by $1.36 \pm 0.29$-fold compared with that in wild-type mice ($P < 0.05$). These indicate that CF6 might increase the expression and release of sFlt-1 in the vessels through acidosis-induced c-Src activation.

## INTRODUCTION

Nitric oxide (NO) and prostacyclin are recognized as the major mediators for maintenance of vascular homeostasis, and the decrease in NO and prostacyclin release is implicated in endothelial dysfunction. Vascular endothelial growth factor (VEGF) is a well-known promoter of angiogenesis. It also induces the generation of NO and prostacyclin in vascular endothelial cells, suggesting a role for VEGF in the regulation of vascular homeostasis.[1,2] VEGF receptor-1 (VEGFR-1), a member of the VEGFR family, binds to VEGF-A, placental growth factor and VEGF-B. An important feature of VEGFR-1 is that, unlike other VEGFR genes, it expresses two types of mRNA, one for a full-length receptor and another for a soluble short protein known as soluble VEGFR-1 (sFlt-1). The binding affinity of VEGFR-1 for VEGF-A is higher than that of VEGFR-2, whereas the kinase activity of VEGFR-1 is weaker than that of VEGFR-2.[3] Therefore VEGFR-1,

especially sFlt-1, plays a negative role in the VEGF signal pathway by trapping the ligand, whereas VEGFR-2 plays a positive role. Overexpression of sFlt-1 was first shown in the placenta of pre-eclamptic patients, and it was suggested to cause the major pathological symptoms such as hypertension and renal dysfunction. Although the factors regulating the synthesis and secretion of sFlt-1 during pregnancy are largely unknown, hypoxia has been shown to increase sFlt-1 production in placental cytotrophoblasts.[4] Angiotensin II (Ang II), a representative proatherogenic molecule, was also reported to induce synthesis and secretion of sFlt-1 by nonhypoxic induction of the hypoxia-inducible transcription factor-$\alpha$ through $AT_1$ receptor pathways, but it was observed only in human trophoblasts, not in vascular endothelial cells.[5,6]

We recently showed that coupling factor 6 (CF6), a component of adenosine triphosphate (ATP) synthase, is present in the systemic

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circulation[7] and suppresses prostacyclin generation through inhibition of cytosolic phospholipase $A_2$[8] and NO generation by upregulating asymmetric dimethylarginine, an endogenous competitive inhibitor of NO synthase.[9] It is of interest that the molecular rotary motor $F_1F_0$ complex, ATP synthase, is present in the plasma membrane,[10,11] and that endogenous prostacyclin and NO inhibitor CF6 forces the backward rotation of $F_0$ motor after binding to $F_1$ motor at the plasma membrane, resulting in intracellular acidosis.[12] This signal is clearly distinct from that of other proatherogenic molecules, such as Ang II, that induce intracellular alkalosis by activation of sodium hydrogen exchanger after binding to each receptor. It is noted that intracellular acidosis is easily caused by hypoxia, and hypoxia is well known to be associated with stimulation of sFlt-1 release.[4] We therefore tested the hypothesis that CF6 upregulates sFlt-1 generation and increase by acidosis-induced c-Src activation in the vascular endothelial cells.

## MATERIALS AND METHODS

### Materials
The HuMedia-EG2 kit was purchased from Kurabo Co. Ltd, Osaka, Japan. The QIAamp RNA kit was purchased from QIAGEN (Tokyo, Japan). Human sFlt-1, VEGFR-2 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) primers, TaqMan probe and TaqMan Gold reverse transcriptase (RT)-PCR kit were obtained from Applied Biosystems (Foster City, CA, USA). Human CF6 was purchased from Phoenix Pharmaceuticals Inc. (Belmont, CA, USA). sFlt-1 enzyme-linked immunosorbent assay (ELISA) kits for human and mouse were purchased from R&D systems Inc. (Minneapolis, MN, USA). Antibodies for human VEGFR-1 and GAPDH were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). PP1, a specific inhibitor of c-Src, was purchased from Biomol International LP (Plymouth Meeting, PA, USA). Polyvinilidene difluoride membrane was from Bio-Rad Laboratories (Hercules, CA, USA). Enhanced chemiluminescence plus detection systems were from Amersham Pharmacia Biotech (Piscataway, NJ, USA). All other reagents were of the finest grade available from Sigma Chemical Co. (St Louis, MO, USA).

### Cell culture
Human umbilical vein endothelial cells (HUVEC) were cultured in HuMedia-EG2 (complete media) at 37 °C under 5% $CO_2$. HUVEC from the second to sixth passage were used for the study. After treatment with various molecules, the viability of the cells, which was determined by Trypan blue excretion, was generally >95%.

### Construction of vectors and generation of transgenic mice
A gene for the N-terminal portion of human calcitonin (Met 1-Arg 84) was fused to a gene for the mature ATP synthase CF6 (Asn 33-Ala 108). The fused gene was subcloned into pNE plasmid, in which the globin promoter of pdKCR-dhfr plasmid[13] was replaced by human elongation factor 1α promoter. The resultant recombinant plasmid was digested with PvuII and Aor51HI to generate 2.8 kb of DNA fragment consisting of the human elongation factor 1α promoter, human calcitonin/CF6 fused gene and SV40 polyA additional sequence. The DNA fragment was then microinjected into the pronuclei of single-cell fertilized mouse embryos to generate TG mice. By using COS cells it was confirmed that the expressed fusion protein was released outside of the cells in the form of mature human CF6 (Asn33-Ala108) after cleavage.

Two lines of homozygous TG mice were produced. The gene expression of CF6 mRNA was increased by twofold in overall tissues in both lines of TG mice. All animals were maintained in the same environment, at the same temperature and humidity and with free access to food and water. Systolic arterial blood pressure at baseline, which was measured by the tail-cuff method under consciousness, was increased in one line of TG mice compared with wild-type (WT) mice aged 15–20 weeks ($120 \pm 5$ vs. $110 \pm 3$ mm Hg, $P<0.05$), but not in another line. All experiments were conducted in TG mice and WT mice aged 10–12 weeks.

### Protocol
Human umbilical vein endothelial cells were treated with CF6 at $10^{-7}$ M for 24 or 48 h with or without various inhibitors for signaling. Further the cells' gene expression of sFlt-1 and VEGFR-2, and the concentration of sFlt-1 in the culture media were measured using real-time RT-PCR or the ELISA kit. The culture supplemented with various inhibitors was incubated for 15 min before the experiments. In another experiment, the concentration of sFlt-1 in the culture media was measured using the ELISA kit after pretreatment with anti-CF6 antibody (1:1000 dilution).

### Determination of gene expression
Total RNA was extracted from the cells using the QIAamp RNA kit. A two-step RT-PCR was carried out according to the protocol supplied with the TaqMan Gold RT-PCR kit. The standard curves of sFlt-1, VEGF-A, intercellular adhesion molecule-1 (ICAM-1), oxidative stress-related genes such as lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), and GAPDH were linear between 0.1 and 250 ng $\mu l^{-1}$ total RNA. Values were averaged from duplicate data and normalized with the human GAPDH.

### Quantification of sFlt-1 protein in cell culture media and plasma
The concentrations of human sFlt-1 in the cell culture supernates and mouse sFlt-1 in the plasma were measured using the specific commercial sandwich ELISA kit according to the manufacturer's protocols.

### Western blot for VEGFR-1 and sFlt-1 proteins
Tissue and cell samples were homogenized in RIPA lysis buffer (20 mmol l$^{-1}$ Tris-HCl, pH 7.5, 150 mmol l$^{-1}$ NaCl, 1 mmol l$^{-1}$ EDTA, 1 mmol l$^{-1}$ EGTA, 1% Triton X-100, 1% glycerol, 1 mmol l$^{-1}$ dithiothreitol and 0.5 mmol l$^{-1}$ phenylmethylsulfonyl fluoride). Supernate samples were partially purified by Sep-Pak C18 cartridges. Protein concentrations of cell lysates and concentrated supernates were determined by the Bradford method. Samples were then mixed with Laemmli buffer containing 5% β-mercaptoethanol, and were evenly loaded onto SDS-polyacrylamide gel electrophoresis. The protein was transferred electrophoretically to a polyvinilidene difluoride membrane. After blocking for 1 h, the membranes were incubated with the primary antibody for VEGFR-1 and GAPDH at 4 °C overnight. The protein bands for antigen–antibody complexes were detected by the enhanced chemiluminescence plus detection systems. Densitometric analysis was performed using Scion image software, and the ratio relative to the protein bands was calculated for each sample.

### Statistics
All data are shown as mean $\pm$ 1s.e.m. One-way analysis of variance for multiple comparisons followed by Bonferroni's test was used for statistical analysis. The level of significance was less than 0.05.

## RESULTS

### Effects of CF6 on gene expression of sFlt-1
As shown in Figure 1a, the ratio of sFlt-1 to GAPDH mRNA by real-time quantitative RT-PCR was increased at 24 h by $1.59 \pm 0.29$-fold ($P<0.05$) using $10^{-7}$ M CF6 and returned to the baseline at 48 h. As shown in Figure 1b, the ratios of sFlt-1 to GAPDH mRNA were increased in a dose-dependent manner after 24-h exposure to CF6 ($P<0.05$, one-way analysis of variance). When the dose of CF6 was increased to $10^{-6}$ M, no further increase in sFlt-1 mRNA was observed (Figure 1b). Figure 1c shows the effects of CF6 at $10^{-7}$ M with or without PP1, an inhibitor of c-Src, and efrapeptin, an inhibitor of ATPase, on the ratios of sFlt-1 to GAPDH mRNA. Pretreatment with PP1 inhibited CF6-induced increase in gene expression of sFlt-1 ($P<0.05$). Efrapeptin at $10^{-5}$ M also dampened CF6-induced increase in gene expression of sFlt-1 ($P<0.05$). As shown in Figure 1d, CF6 at $10^{-7}$ M did not affect the gene expression of VEGFR-2 and VEGF-A, but increased the expression of ICAM-1 and LOX-1. As reported previously,[12] CF6 at $10^{-7}$ M activated ATPase activity at the surface of vascular endothelial cells and led to intracellular acidosis, resulting in
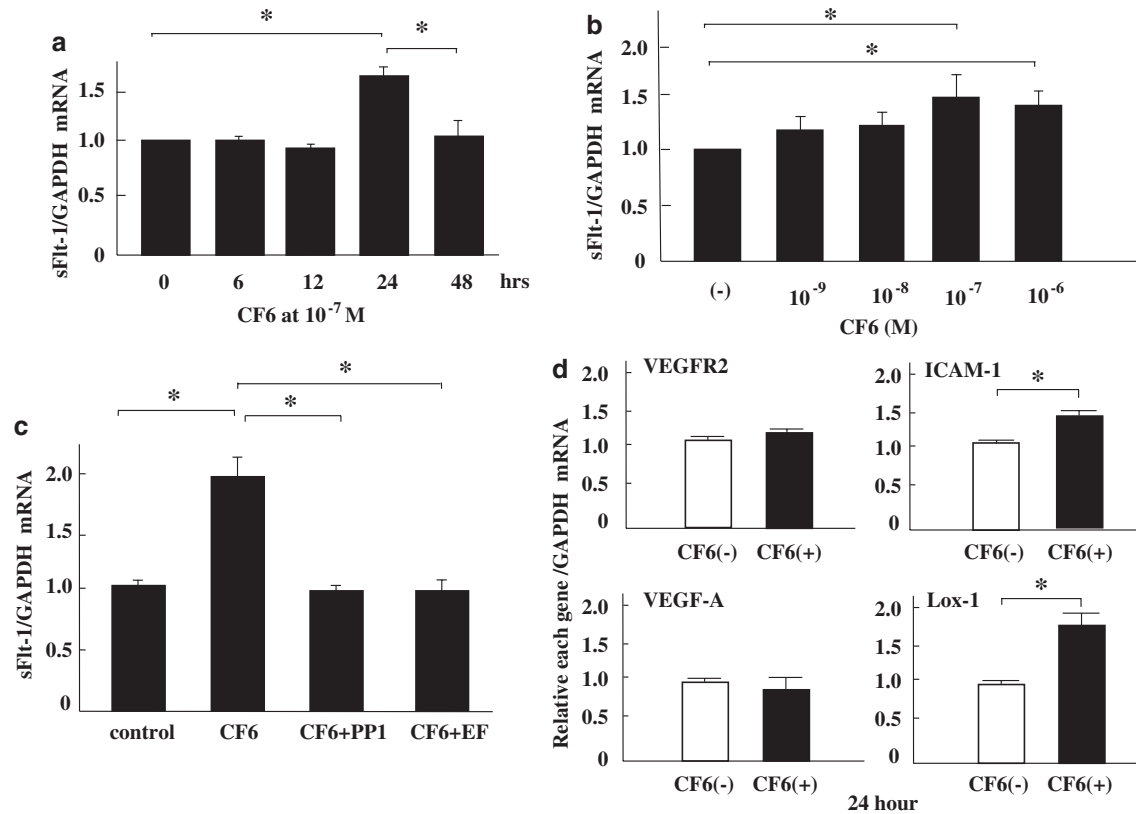
Figure 1 Effects of human coupling factor 6 (CF6) on the gene expression of sFlt-1 and vascular endothelial growth factor receptor-2 (VEGFR-2) in human umbilical vein endothelial cells. (a) Chronological changes after exposure to CF6 at $10^{-7}$ M ($n=6$). *$P<0.05$. (b) Dose-related changes after 24-h exposure to CF6 at $10^{-9}$–$10^{-6}$ M ($n=3$–7). *$P<0.05$. (c) Effects of CF6 on the ratios of sFlt-1 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA at 24 h and effects of various compounds on CF6-induced increase in sFlt-1 gene expression ($n=4$–6). *$P<0.05$. Control (without agonists), CF6 at $10^{-7}$ M, PP1 at 50 μM, EF: efrapeptin at $10^{-5}$ M. (d) Effect of CF6 on the ratios of VEGFR-2, vascular endothelial growth factor (VEGF)-A, intercellular adhesion molecule-1 (ICAM-1) and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) to GAPDH mRNA at 24 h.

activation of c-Src. Efrapeptin blocked CF6-induced intracellular acidosis and c-Src activation, whereas PP1 blocked c-Src activation without affecting induction of intracellular acidosis (data not shown).

### Effects of CF6 on sFlt-1 protein release

As shown in Figure 2a, the release of sFlt-1 protein in the supernate was increased in a time-dependent manner (both $P<0.05$, one-way analysis of variance). CF6 at $10^{-7}$ M enhanced the release of sFlt-1 protein, and the level at 48 h was significantly increased by $1.72\pm0.24$-fold ($P<0.05$) compared with control. When the dose of CF6 was increased to $10^{-6}$ M, no further increase in the release of sFlt-1 was observed (Figure 2b). Figure 2c shows the effect of CF6 at $10^{-7}$ M with or without PP1 and efrapeptin on the release of sFlt-1 protein in HUVEC. Pretreatment with PP1 inhibited CF6-induced increase in the release of sFlt-1 protein ($P<0.05$) without affecting its basal release (PP1(−) $221\pm7$ vs. PP1(+) $200\pm4$ pg ml$^{-1}$, $P=$NS). Efrapeptin at $10^{-5}$ M also dampened CF6-induced increase in the release of sFlt-1 protein ($P<0.05$) without affecting its basal release (efrapeptin (−) $221\pm7$ vs. efrapeptin (+) $213\pm4$ pg ml$^{-1}$, $P=$NS). As shown in Figure 2d, pretreatment with anti-CF6 antibody inhibited CF6-induced increase in the release of sFlt-1 protein ($P<0.05$) without affecting its basal release.

### Effects of CF6 on sFlt-1 and VEGFR-1 proteins

Figure 3a illustrates the representative bands for sFlt-1 and VEGFR-1 proteins in the presence or absence of CF6 at $10^{-7}$ M for 48 h. The immunoreactive bands for sFlt-1 at 60 kDa and VEGFR-1 at 180 kDa were both increased after treatment with CF6 to similar degrees. As shown in Figure 3b, the ratios of sFlt-1 and VEGFR-1 to GAPDH protein were increased by $52\pm13$ and $60\pm11\%$, respectively, in HUVEC (both $P<0.05$).

### Plasma concentration of sFlt-1 in TG mice

As shown in Figure 4, the plasma level of sFlt-1 was increased in TG mice than in WT mice ($5.23\pm0.58$ vs. $3.83\pm0.86$ ng ml$^{-1}$, $P<0.05$).

## DISCUSSION

### CF6 increases expression and release of sFlt-1

sFlt-1 protein is upregulated by hypoxia through hypoxia-inducible transcription factor-α in the trophoblasts.[14–16] Ang II, a representative proatherogenic molecule, was also reported to induce the synthesis and secretion of sFlt-1 by nonhypoxic induction of hypoxia-inducible transcription factor-α through AT$_1$ receptor pathways,[5,6] but it was observed only in human trophoblasts, and not in vascular endothelial cells. We recently showed that CF6 suppresses prostacyclin and NO generation, and that its effect is mediated by intracellular acidosis.[11] As intracellular acidosis is easily caused by hypoxia, and hypoxia is the only known stimulator of sFlt-1, we investigated the role of CF6 in the expression of sFlt-1. As expected, the results showed that CF6 significantly increased the gene and protein expression of sFlt-1 in HUVEC, but it was not remarkable. We therefore investigated whether
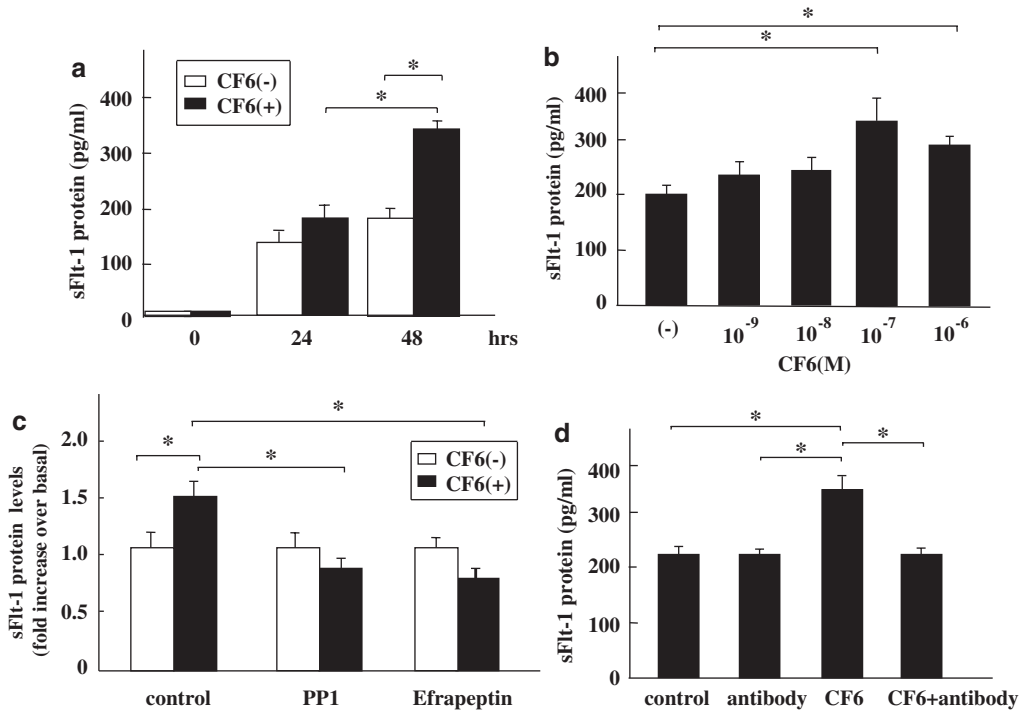
**Figure 2** Effects of human coupling factor 6 (CF6) on the release of sFlt-1 protein in human umbilical vein endothelial cells. (**a**) Chronological changes after exposure to $10^{-7}$ M CF6 ($n=6$). *$P<0.05$. (**b**) Dose-related changes after 48-h exposure to $10^{-9}$ to $10^{-6}$ M CF6 ($n=6$–8). *$P<0.05$. (**c**) Effects of various compounds on CF6-induced sFlt-1 protein release ($n=6$). *$P<0.05$. Control (without agonists), $10^{-7}$ M CF6, 50 μM PP1, EF: $10^{-5}$ M efrapeptin. (**d**) Effects of anti-CF6 antibody on CF6-induced sFlt-1 protein release ($n=6$). *$P<0.05$. Control (without anti-CF6 antibody), $10^{-7}$ M CF6, anti-CF6 antibody (1:1000 dilution).
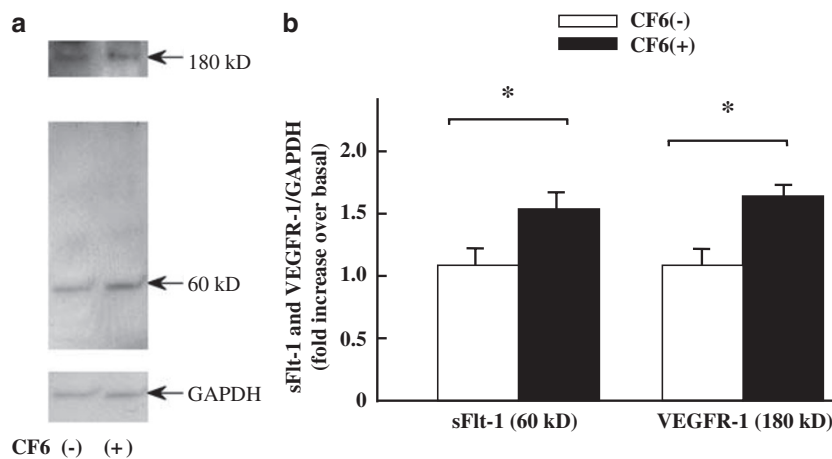


**Figure 3** Effects of human coupling factor 6 (CF6) on the expression of sFlt-1 and vascular endothelial growth factor receptor-1 (VEGFR-1) protein in human umbilical vein endothelial cells. (**a**) Representative bands for sFlt-1 and VEGFR-1 after 48-h exposure to $10^{-7}$ M CF6. (**b**) Ratios of sFlt-1 and VEGFR-1 to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) protein after 48-h exposure to $10^{-7}$ M CF6 ($n=4$).

this increase in sFlt-1 affects the gene expression of ICAM-1 and LOX-1. We found that CF6 at $10^{-7}$ M increased the expression of ICAM-1, which is related to the vascular endothelial function and the oxidative stress-related gene LOX-1. We further showed that CF6 suppressed endothelial NO synthase activity by increasing release of asymmetric dimethylarginine and decreasing phosphorylation at Ser[1177].[9,17] These observations suggest that CF6 affects the endothelial function through various mechanisms, and that the CF6-induced increase in sFlt-1 is operative to the cells in the present culture condition.

sFlt-1 and VEGFR-1 proteins are derived from alternative splicing of the same mRNA, and this process has been identified as a key regulatory step in production of sFlt-1.[18] Both VEGFR-1 and sFlt-1 proteins were proportionally increased in HUVEC stimulated with CF6. Therefore, it is reasonable to presume that upregulation of sFlt-1 does not occur at the level of alternative splicing but at the level of transcription or mRNA stability. It is of importance that CF6 did not affect the gene expression of VEGF-A. Therefore, the ratio of sFlt-1 to VEGF-A seems to be increased by CF6, thereby resulting in the progression of atherosclerosis.
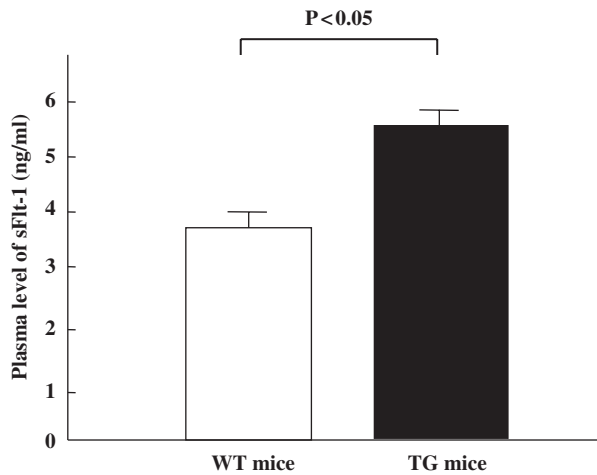
**Figure 4** Plasma level of sFlt-1 in transgenic (TG) mice and wild-type (WT) mice.

The present results show that CF6 increased the gene and protein expression of sFlt-1, but it was significant only at concentrations $> 10^{-7}$ M. Similar to other vasoactive substances, the plasma level of CF6 ($10^{-9}$–$10^{-8}$ M) was lower than the critical concentration ($10^{-7}$ M) at which sFlt-1 expression in the cultured vascular endothelial cells was increased *in vitro*. We previously showed that intravenous administration of anti-CF6 antibody to the rats counteracted its biological effects.[7] Therefore, CF6 is active *in vivo* at lower concentrations compared with the effective one *in vitro*. CF6 seems to function as a novel stimulator of sFlt-1 *in vivo*.

We clearly showed that the plasma level of sFlt-1 was increased in TG mice. However, it is unclear whether the circulating sFlt-1 is mainly derived from vascular endothelial cells. We chose human elongation factor $1\alpha$ as the promoter of the N-terminal portion of human carcitonin/CF6 fused gene in order to achieve CF6 gene expression in overall tissues, including the cardiovascular system. Indeed, it was confirmed that the gene expression of CF6 is upregulated by twofold in overall tissues in TG mice. Further examination is needed to determine the origin of the circulating sFlt-1 in TG mice. In contrast, the TG mice manifested the phenotype of elevation of systolic arterial blood pressure. Therefore, it is likely that cardiovascular risk is increased in TG mice.

### Regulation mechanism for sFlt-1 gene expression
The tyrosine kinase c-Src is a key enzyme regulating a number of downstream effectors related to vasoconstriction.[19] We recently showed that CF6 stimulates ATPase activity at the cell surface of HUVEC after binding to the $\beta$-subunit of plasma membrane ATP synthase and induces intracellular acidosis by a transient flux of hydrogen ion through $F_0$.[12] As c-Src becomes activated by acidification,[18] we previously investigated the linkage of CF6 to c-Src activation and showed that CF6 activated c-Src in HUVEC.[17] Using PP1, a specific inhibitor of c-Src, and efrapeptin, a specific inhibitor for ATPase, we examined whether c-Src contributes to the increase in sFlt-1 expression in HUVEC. Efrapeptin inhibited the CF6-induced increase in sFlt-1 expression by blocking intracellular acidosis and c-Src activation. PP1 inhibited the CF6-induced increase in sFlt-1 expression by blocking c-Src activation without affecting induction of intracellular acidosis.[17] These suggest that CF6 increased the gene and protein expression of sFlt-1 through intracellular acidosis and c-Src activation in HUVEC.

It was reported that treatment of endothelial cells with statins upregulated the release of heme oxygenase-1 and inhibited the release of sFlt-1.[20] The addition of simvastatin, fluvastatin or mevastatin significantly decreased the basal production and VEGF-E-induced release of sFlt-1 from endothelial cells. As statins counteract against the production of reactive oxygen species, the release of sFlt-1 may be associated with the redox-sensitive signaling pathway. It is proposed that redox state *per se* may be involved in sFlt-1 expression upstream of c-Src in vascular endothelial cells. Further examination is needed to clarify this.

### Implications of linkage between CF6 and sFlt-1 in cardiovascular disorders
In addition to its inhibitory effects on prostacyclin and NO,[8] CF6 was first shown to be involved in endothelial dysfunction by increasing the release of sFlt-1. The plasma level of sFlt-1 is increased in patients with hypertension and acute coronary syndromes,[21] and these states are closely related to the presence of endothelial dysfunction. It was reported that the administration of recombinant sFlt-1 or a VEGF-neutralizing antibody results in glomerular endothelial cell damage and proteinuria in rats,[22] and adenoviral delivery of sFlt-1 to pregnant rats mimics the clinical manifestations of preeclampsia.[23] These suggest that the excess circulating sFlt-1 plays a role in the pathogenesis of atherosclerosis.

In clinical settings, the plasma level of CF6 was increased in human hypertension,[24] end-stage renal disease[25] and type 2 diabetes mellitus[26] by 1.5–3.0-fold compared with that in normal subjects. Furthermore, it was modulated by salt intake, and the percent change in plasma CF6 level was negatively correlated with that in plasma NO metabolite level.[24] In patients with end-stage renal disease, the plasma level of CF6 was related to the development of ischemic heart disease.[25] All these suggest that CF6 plays an important role in the genesis and progression of cardiovascular disorders, operating as a potent proatherogenic molecule. As CF6 generation is regulated by the nuclear factor-κB signaling pathway,[27–29] CF6 seems to be associated with the development of atherosclerosis synergistically with other inflammatory compounds that are regulated by nuclear factor-κB signaling.

In conclusion, this report first shows that CF6 might increase sFlt-1 release through the activation of c-Src in vascular endothelial cells. As sFlt-1 antagonizes the effect of VEGF as a promoter of angiogenesis, NO and prostacyclin, CF6 may be involved in the regulation of vascular homeostasis under physiological and pathological conditions. In light of these findings, CF6 seems to play an important role in the initiation and progression of atherosclerosis by the complex mechanisms including sFlt-1.

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