Full Title

Suppressed Soluble Fms-Like Tyrosine Kinase-1 Production Aggravates Atherosclerosis in CKD : Evaluation by Circulating sFlt-1 Levels after Low-Dose Heparin Injection

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Running Title

Role of sFlt-1 in CKD-Associated Atherosclerosis

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Abstract

The pathway initiated by placental growth factor (PlGF) via its receptor, fms-like tyrosine kinase (Flt-1), is involved in the pathogenesis of atherosclerosis, but its role remains unknown in worsening of atherosclerosis in chronic kidney disease (CKD). A soluble isoform of Flt-1 (sFlt-1), which antagonizes the biological effects of PlGF, exists both in the circulation and on the surface of endothelial cells. Thus, sole measurement of plasma sFlt-1 levels is not sufficient to understand the pathophysiological role of sFlt-1 in CKD. We measured plasma sFlt-1 levels in 291 CKD patients and 52 control subjects. Baseline sFlt-1 levels were negatively correlated with estimated glomerular filtration rate (r=–0.323, P<0.001). However, intravenous injection of low-dose heparin (0.4 IU/Kg) increased sFlt-1 levels in correlation with renal function and consequently reversed this correlation into higher positive fashion (r=0.537, P<0.001). The post-heparin ratio of PlGF to sFlt-1 was correlated with severity of coronary atherosclerosis, and an independent predictor of cardiovascular events (hazard ratio 2.849, P=0.006), while the pre-heparin ratio was not. Genetic blockade of sFlt-1 exaggerated atherosclerosis in $ApoE^{-/-}$ mice. In conclusion, decreased production of sFlt-1 derived from renal dysfunction accelerates the proatherogenic bioactivity of PlGF in CKD patients, and post-heparin levels of sFlt-1 possibly represent the total production of sFlt-1 in vivo.

Keywords

chronic kidney disease; atherosclerosis; soluble fms-like tyrosine kinase-1, placental growth factor

INTRODUCTION

The growing number of patients with chronic kidney disease (CKD) has become an emerging global public health problem. Previous epidemiologic studies have revealed that CKD patients are not only at high risk of developing end-stage renal disease, but are also at drastically higher risk of cardiovascular morbidity and mortality.[1-6] In addition, the incidence of adverse cardiovascular events progressively increases with worsening renal dysfunction.[7] Despite significant effort, the molecular mechanisms linking renal dysfunction and development of cardiovascular disease have not been fully clarified.

VEGF has been recognized as a key regulator of angiogenesis and vascular permeability in the kidney, while the pathophysiological role of placental growth factor (PlGF), a member of VEGF family, is unclear in CKD patients. Recently, evidence is accumulating that the signaling pathway initiated by PlGF through its receptor, fms-like tyrosine kinase-1 (Flt-1), is involved in the pathogenesis of atherosclerotic diseases and cardiovascular events.[8-11] PlGF selectively and specifically binds with Flt-1 and accelerates atherosclerotic processes through enhancement of intramural angiogenesis and monocyte recruitment.[12-15] Recruited monocytes differentiate into macrophages, are further activated via PlGF/Flt-1 signal transduction in macrophages and produce proinflammatory cytokines such as tumor necrosis factor-alpha (TNFα) and monocyte chemoattractant protein-1 (MCP-1), resulting in augmentation of inflammatory response and plaque vulnerability.[16,17] A soluble isoform of Flt-1 that is produced by alternative splicing of primary, full-length Flt-1 mRNA consists of extracellular domains with PlGF- and heparin-binding motifs and acts as an intrinsic inhibitor of PlGF, that is, as a decoy protein.[18-20]

We previously reported that when plasma samples were collected after the establishment of arterial blood access with heparinized saline flush in patients undergoing cardiac catheterization, decease in estimated glomerular filtration rate (eGFR) was significantly associated with lower plasma levels of sFlt-1, and that the coronary atherosclerosis was aggravated in patients with higher PlGF/sFlt-1 ratio.[21] Based on these findings, we hypothesized that balance between PlGF and sFlt-1 is maintained in healthy subjects, whereas this balance is disturbed by down-regulation of sFlt-1 in CKD patients, which leads to relative activation of atherogenic PlGF signal through Flt-1, as schematically illustrated in Figure 1. However, another clinical research has shown that sFlt-1 levels were not decreased in patients with renal dysfunction,[22] thus sFlt-1 levels in CKD are still controversial.

Recent biochemical and biological studies have revealed that sFlt-1 exists not only in a circulating form in peripheral blood, but is also stored on the surface of endothelial cells, possibly by binding to heparan sulfate proteoglycans which is the component of the extracellular matrix,[23,24] based on the following: plasma sFlt-1 levels are elevated by heparin injections in humans, and sFlt-1 is released from mouse aortic organ cultures by heparin.[25-28] Thus, measurement of plasma sFlt-1 alone may not be sufficient to evaluate its entire PlGF-antagonizing effect or to understand its role in the cardiorenal connection.

Here we present a heparin loading test for the measurement of plasma sFlt-1 levels. Using this loading test, we found that post-heparin plasma sFlt-1 levels are a possible surrogate marker for the total amounts of both circulating and stored sFlt-1, and that the post-heparin PlGF/sFlt-1 ratio is a predictor of advanced atherosclerosis and poor prognosis in CKD patients. Combining clinical findings with experimental data derived

from genetically engineered mice that lack sFlt-1 but retain the full length Flt-1 receptor, we confirm that sFlt-1 is not merely a biomarker of endothelial damage but is involved in the pathogenesis of aggravated atherosclerosis in patients with CKD.

RESULTS

Heparin loading test

We first studied the effect of intravenous bolus injection of heparin on plasma levels of sFlt-1, PlGF, and VEGF in 8 subjects (4 control subjects, and 4 patients with CKD5). Plasma sFlt-1 levels were dose-dependently increased after heparin injection in all patients (Figure 2a). Next, we studied the time course of plasma sFlt-1 levels after heparin injection at a dose of 0.4 IU/kg (Figure 2b). Plasma sFlt-1 levels peaked at 5 minutes after heparin injection in both patient groups, and peak concentrations of sFlt-1 were significantly lower in CKD5 patients than in control subjects. Based on these results, we adopted a "heparin loading test" protocol using a dose of 0.4 IU/kg, and measured plasma levels of sFlt-1 before and 5 minutes after heparin injection in the following clinical investigation.

Post-heparin sFlt-1 levels are decreased in CKD patients

We studied 343 subjects (males: 59%; median age, interquartile range: 69 years, 60 to 76 years), including 52 control subjects as well as 291 CKD patients who had an eGFR below 60 ml/min/1.73 m^2 and/or continuous proteinuria over 3 months. Of the 291 CKD patients, 70 were receiving maintenance hemodialysis. Table 1 shows the baseline characteristics of all patients stratified by CKD stage.

We first assessed the relation between renal function (eGFR) and (1) plasma levels

of sFlt-1 and PlGF, and (2) serum levels of VEGF, in 273 patients. Hemodialysis patients were excluded from this evaluation because eGFR values are not accurate in this population. Baseline plasma levels of sFlt-1 were negatively correlated with eGFR (r=–0.323, P<0.001) (Figure 3a). To our surprise, heparin injection (0.4 IU/kg) reversed this relationship, resulting in a highly positive correlation (r=0.537, P<0.001) (Figure 3b). Baseline PlGF levels increased with decreasing eGFR (r=–0.479, P<0.001), but PlGF levels did not change after heparin injection (Figure 3c,d). VEGF levels did not correlate with eGFR either before or after heparin injection (r=–0.032, P=0.597; r=–0.065, P=0.284, respectively) (Figure 3e,f).

Because the proatherogenic activity of PlGF is counterbalanced by sFlt-1, we examined the relation between PlGF/sFlt-1 ratio and eGFR. As expected, a highly negative correlation was observed (r=–0.616, P<0.001) (Figure 3g). A correlation between post-heparin PlGF/sFlt-1 ratio and CKD stage was also seen, even when hemodialysis patients were included (Figure 3h).

Post-heparin PlGF/sFlt-1 ratio is a predictor of cardiovascular events

A total of 38 cardiovascular events had occurred during a median follow-up period of 177 days. Subjects were divided into 2 groups according to the median values of the pre- and post-heparin PlGF/sFlt-1 ratios. Although the pre-heparin PlGF/sFlt-1 ratio was not related to the incidence of cardiovascular events (P=0.107) (Figure 4a), patients with a higher post-heparin PlGF/sFlt-1 ratio had a significantly higher incidence of cardiovascular events compared to those with a lower post-heparin PlGF/sFlt-1 ratio (P=0.001) (Figure 4b). Additionally, after the univariate analysis, multivariate analysis using Cox proportional hazard regression revealed that the

post-heparin PlGF/sFlt-1 was an independent predictor of cardiovascular events. (hazard ratio 2.849 [95% CI, 1.350–6.015], P=0.006) (Table 2).

Expression levels of sFlt-1 are decreased in a CKD animal model

Our preliminary study showed that baseline sFlt-1 mRNA expression levels were dominant in lung and aortic tissue in wild-type (WT) mice (Supplementary Figure 1a). We also revealed that sFlt-1 was expressed in endothelial cells, smooth muscle cells which are the component of vascular tissues (Supplementary Figure 1b). Based on this finding, changes in mRNA expression levels were investigated using lung, aorta, and kidney tissues in mice with renal dysfunction. Remarkably, a reduction in the expression levels of sFlt-1 mRNA was seen in both lung and kidney in 5/6 nephrectomized apolipoprotein E-deficient ($ApoE^{-/-}$) mice compared to control $ApoE^{-/-}$ mice (Table 3). This finding indicates that in the setting of renal dysfunction, the production of sFlt-1 was decreased not only in kidney, but possibly in the entire vascular system. On the contrary, mRNA expression levels of PlGF and VEGF were significantly increased in 5/6 nephrectomized $ApoE^{-/-}$ mice compared to control $ApoE^{-/-}$ mice.

sFlt-1 is stored on arterial tissue and released by heparin

Injection of heparin into the tail veins of WT mice induced a 7-fold increase in sFlt-1 plasma levels (Figure 5a). Thoracic aorta from WT mice were cultured in and ex vivo system for 24 hours in the presence or absence of heparin to investigate whether sFlt-1 is displaceable by heparin. sFlt-1 was found to be released into cultured medium after heparin treatment (Figure 5b). Furthermore, cultured Human

Microvascular Endothelial Cells (HMVECs) released sFlt-1 into medium in a dose-dependent fashion following 10 minutes of treatment with increasing concentrations of heparin (Figure 5c), but heparin treatment did not alter sFlt-1 mRNA expression levels in HMVECs (Figure 5d). These findings indicated that heparin treatment did not stimulate sFlt-1 production, but released membrane-associated sFlt-1 into conditioned medium.

Plasma sFlt-1 levels in sFlt-1$^{-/-}$ mice were approximately half those in WT mice, although mRNA levels were completely abrogated (Supplementary Figures 2, 3a, b). Plasma and mRNA levels of PlGF and VEGF and the vessel density did not differ between sFlt-1$^{-/-}$ and WT mice (Supplementary Figures 3c-f, 4a-f). Heparin injection increased plasma levels of sFlt-1 in both sFlt-1$^{-/-}$ and WT mice, but the magnitude of the post-injection increase was markedly lower in sFlt-1$^{-/-}$ mice than WT mice (Figure 5a). As expected, similar results were obtained in ex vivo experiments with thoracic aorta from Flt-1$^{-/-}$ mice (Figure 5b). These findings indicated that the smaller increase in sFlt-1 after heparin injection was the result of a decrease in the total amount of sFlt-1 production, both in patients with CKD and in a murine model of the disease.

Endothelial damage and oxidative stress is associated with sFlt-1 reduction in CKD

To investigate the mechanisms underlying the decrease in sFlt-1 expression in patients with CKD, cultured Human Umbilical Artery Endothelial Cells (HUAECs) were incubated with serum from CKD patients. Expression levels of sFlt-1 mRNA were significantly decreased in cultures with serum from CKD5 and CKD5D compared to

control (Figure 6a), while PlGF mRNA levels were increased in cultures with serum from CKD5 and CKD5D (Figure 6b). There were no significant differences in expression levels of VEGF mRNA among the 3 groups (Figure 6c). Expression levels of endothelial damage markers (selectin, ICAM1, and VCAM1) were significantly higher in cultures with serum from CKD5 and CKD5D than with serum from control (Figures 6d-f).

Expression levels of heme oxygenase-1 (HO-1) were also increased in HUAECs treated with serum from CKD5 and CKD5D compared to those treated with serum from control (Figure 7a). Moreover, intracellular reactive oxygen species (ROS) production was significantly enhanced in cultures with serum from CKD5D as opposed to control and CKD5 (Figure 7b). The addition of vitamin E to the culture medium blunted the decrease in sFlt-1 and reversed the increase in PlGF and in the CKD5D group (Figures 7c, d). Next, we harvested lung and kidney from 5/6 nephrectomized ApoE$^{-/-}$ mice and measured the expression levels of HO-1 and NADPH-oxidase (Nox-2) mRNA using quantitative real-time reverse transcription polymerase chain reaction (RT-PCR). Expression of oxidative stress–related proteins was increased in both lung and kidney in nephrectomized ApoE$^{-/-}$ mice (Figures 7e–h). These findings indicate that the enhanced redox state of plasma in patients with CKD plays a role in reducing the expression of sFlt-1 mRNA as well as in the development of endothelial damage.

Atherosclerosis is aggravated in sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice

To investigate whether sFlt-1 depletion per se promotes atherosclerosis, we generated sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice. As shown in Table 4, sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice had lower LDL levels compared to control ApoE$^{-/-}$ mice, but other parameters, including

serum creatinine, were comparable between the 2 groups, demonstrating that the results of this animal experiment were not affected by renal dysfunction.

The total aortic plaque area of sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice was significantly larger than that of sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice (Figures 8a, b, g). The atherosclerotic lesion area at the aortic valve was also larger in sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice than in sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice (Figures 8c, d, h). More F4/80-positive cells were seen in the atherosclerotic plaque area in sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice than in sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice (Figures 8e, f, i). Specific reduction of sFlt-1 therefore induced progression of atherosclerosis and macrophage infiltration into atherosclerotic lesions, independently of renal dysfunction, possibly by enhancing the biological activity of PlGF.

Discussion

The present study demonstrated that intravenous injections of small doses of heparin increased plasma sFlt-1 levels and changed the correlation between plasma levels of sFlt-1 and renal function from negative to positive. That is, prior to heparin injection, plasma sFlt-1 levels increased with decreasing eGFR, but after heparin injection these levels decreased with decreasing eGFR. Given that sFlt-1 circulates in the blood and is also stored on the surface of endothelial cells, and that heparin releases the stored sFlt-1, post-heparin plasma levels of sFlt-1 represent the sum of both circulating and released sFlt-1. In addition, the change in correlation pattern caused by the heparin-induced release of sFlt-1 is larger in patients with preserved renal function than patients with renal dysfunction.

In the present study, we confirmed that sFlt-1 mRNA expression was downregulated in the lung and kidney, both of which are major sFlt-1–producing organs, in a murine

model of CKD. Furthermore, endothelial expression of sFlt-1 was inhibited in cultured HUAECs by addition of human serum from patients with CKD5 and CKD5D, thus these findings support the hypothesis that endothelial production of sFlt-1 is suppressed in the setting of renal dysfunction. Taking these results into account, pre-heparin plasma sFlt-1 levels are not associated with the production of sFlt-1, but post-heparin levels seem to be a good marker for total body production of sFlt-1 as well as the total capacity to antagonize PlGF bioactivity. Thus, we propose in Figure 9 the hypothetical localization of sFlt-1 before and after heparin treatment in cases of normal and reduced renal function. That is, in CKD, although sFlt-1 production is lower and less is stored on endothelial cells, a relatively larger percentage of sFlt-1 is circulating in plasma. In contrast, in non-CKD cases, although sFlt-1 is produced and stored in more abundance, a relatively smaller percentage is circulating. Heparin treatment releases sFlt-1 from endothelial cells, and the heparin-induced increase in plasma sFlt-1 is larger in non-CKD cases than in CKD.

What are the mechanisms underlying the reduced sFlt-1 production, storage, and release from endothelial cells in CKD? As mentioned above, the addition of serum from patients with CKD5 and CKD5D reduced sFlt-1 expression in cultured HUVECs, accompanied by up-regulation of biomarkers for endothelial injury. Given that ROS production is augmented in CKD patients,[29,30] it is likely that ROS causes endothelial injury and consequently not only inhibits sFlt-1 production but also decreases its storage on endothelial cells. This scenario is supported by the present finding that the addition of serum from patients with CKD5 and CKD5D increased ROS production in HUAEC cultures and that this was blocked by vitamin E. However, further studies are necessary to clarify the involvement of uremic toxins such as indoxyl sulfate, p-cresol,

and other unknown substances that induce endothelial injury by various mechanisms.[31,32] Given that sFlt-1 acts as a decoy receptor to functionally antagonize PlGF action, plasma sFlt-1 levels are assumed to be a biomarker of anti-atherosclerotic or anti-inflammatory conditions. Several earlier works showed that levels of plasma sFlt-1 in the absence of heparin treatment were associated with the severity of underlying disease and with adverse outcomes in patients with heart failure, acute coronary syndrome, hemodialysis, or sepsis.[24,33-35] The investigators in these studies therefore speculated that pre-heparin plasma sFlt-1 levels are a surrogate marker of underlying endothelial injury rather than of anti-atherosclerotic status.

Another possible explanation for the blunted release of sFlt-1 from endothelial cells in CKD is the suppression of sFlt-1 production, independent of any impairment in the sFlt-1 storage capacity of the endothelial surface associated matrix. To evaluate this hypothesis, we generated mutant mice lacking sFlt-1 but normally expressing full length Flt-1 by knocking in the mutated gene that directly binds exon 13 to exon 14 and thus preventing alternative splicing at intron 13. The mice developed normally and had unimpaired renal function. None of their tissues showed the presence of sFlt-1 mRNA, but sFlt-1–like immunoreactivity was detected in plasma at about half the levels seen in WT mice. Recently, other splicing variants of Flt-1 generated by vascular smooth muscle cells, which use a splice acceptor site within intron 14, or other soluble isoforms generated by shedding of the extracellular domain of Flt-1, have been reported.[25,26,36] Based on these previous findings, it is possible that substantial dose of sFlt-1 is generated by unknown alternative splicing mechanism or enzymatic proteolytic process in our animal model. Next, we investigated the clinical significance of post-heparin levels of plasma sFlt-1. As mentioned above, pre-heparin

levels of plasma sFlt-1 are a possible surrogate marker for endothelial injury, while post-heparin levels are a potential surrogate marker for the total capacity to antagonize PlGF bioactivity. In fact, post-heparin sFlt-1 levels were significantly associated with the severity of coronary disease and tended to become a predictor of cardiovascular events, although pre-heparin sFlt-1 levels did not (Supplementary Figures 5a–d). In terms of clinical significance, the post-heparin PlGF/sFlt-1 ratio was associated with severity of coronary disease, and Kaplan-Meier analysis showed that the post-heparin ratio was a significant predictor for cardiovascular events while the pre-heparin ratio was not. Comparing plasma sFlt-1 levels with the plasma PlGF/sFlt-1 ratio, the latter is a stronger predictor for cardiovascular events than the former, probably because it more closely reflects the pro-atherosclerotic condition in CKD. It is reasonable that post-heparin sFlt-1 levels or the PlGF/sFlt-1 ratio are not merely surrogate biomarkers for cardiovascular events, but rather biomarkers that reflect a cause-and-effect relationship in the aggravation of atherosclerosis in CKD.

We previously reported that replacement administration of recombinant human sFlt-1 ameliorated the worsening of atherosclerosis observed in Apo $E^{-/-}$ mice with 5/6 nephrectomy, a CKD model.[21] To further confirm the anti-atherosclerotic properties of sFlt-1, we crossed sFlt-$1^{-/-}$ mice with Apo $E^{-/-}$ mice. As far as we were able to determine, sFlt-$1^{-/-}$ Apo$E^{-/-}$ mice developed normally. Plaque formation and macrophage infiltration were aggravated in sFlt-$1^{-/-}$ Apo$E^{-/-}$ mice, whose renal function was normal, compared with WT mice, clearly indicating that reduction of sFlt-1 production per se could worsen atherosclerosis.

To investigate what is the target cell of angiogenic PlGF signaling, we studied the mRNA expression of Flt-1 in vitro and in vivo. As previously reported,[37,38] we also

confirmed Flt-1 mRNA was expressed not only in endothelial cells, but in vascular smooth muscle cells, and monocytes (Supplementary Figure 1b). Interestingly, TNFα and MCP-1 mRNA levels in peritoneal macrophage obtained from sFlt-1 deficiency were increased compared with cells obtained from WT mice (Supplementary Figure 6a,b). These results indicate macrophage is a possible target of PlGF signaling to aggravate the atherosclerosis in this model.

sFlt-1 antagonizes both PlGF and VEGF signaling, and it is therefore possible that VEGF is also involved in the exacerbation of atherosclerosis in CKD patients. In this study, plasma PlGF levels showed a significant increase with decreasing eGFR, whereas serum VEGF levels did not correlate with eGFR, and did not change by heparin injection. However, VEGF is an established proinflammatory mediator which promotes the development and the progression of atherosclerosis, so further study is needed to elucidate the pathophysiological role of VEGF for CKD associated atherosclerosis.

In conclusion, the present study demonstrates that endothelial production of sFlt-1, which can be assumed by injection of low-dose heparin, is reduced in CKD patients, partly via oxidative stress–dependent mechanisms. The reduction of the capacity to antagonize PlGF promotes atherosclerotic processes that lead to poor prognosis in CKD patients. sFlt-1 thus plays a causal role in the development of aggravated atherosclerosis in patients with CKD.

METHODS

Clinical study

This clinical study was performed using 52 control subjects and 291 patients with

CKD who were admitted to Nara Medical University Hospital between June 22, 2010 and July 30, 2011. All control subjects had an eGFR of 60 ml/min/1.73 m^2 or more, and none had proteinuria. Exclusion criteria were the present use of heparin, present infection, malignancy, acute critical illness (e.g., acute heart failure, acute myocardial infarction, and stroke), pregnancy, age less than 18 years, and unwillingness to participate. The median follow-up was 177 days, and a total of 38 cardiovascular events were occurred. All events were confirmed through medical records in participants who returned to the hospital every one to three months, and through self-reporting by otherwise with telephone. Detailed information is described in Supplementary methods.

Blood Sampling and biomarker measurement

Blood samples were collected before and 5 minutes after intravenous injection of 0.4 IU/kg heparin. Plasma levels of sFlt-1 and PlGF and serum levels of VEGF were measured with commercial sandwich enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, MN, USA).

Clinical end points

The cumulative incidence of cardiovascular events was investigated from the day of examination with a median follow-up period of 177 days. There was no loss of follow-up in any patient. Cardiovascular events were the composite as described in Supplementary methods. This clinical study was approved by the hospital ethics board and written informed consent was obtained from each participant.

Experimental study

Mouse models of renal dysfunction

ApoE$^{-/-}$ mice (C57BL/6 background) were purchased from Taconic Farms (Hudson, NY, USA), and maintained with a standard chow diet until 11 weeks old. Thereafter, mice were maintained on a Western diet (16.5% fat, 1.25% cholesterol, 0.5% sodium cholate) until 22 weeks old. At 8 weeks of age, ApoE$^{-/-}$ mice were randomly assigned to a 5/6 nephrectomy or a control group. 5/6 nephrectomy was performed under anesthesia. Briefly, the right kidney and two thirds of the other kidney were subsequently removed as described previously.[39,40]

Generation of sFlt-1$^{-/-}$ and sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice

We created constitutive sFlt-1 knockout mice in which intron 13 of the sFlt-1 gene was replaced by a neomycin resistance gene and directly connect exon 13 to exon 14. Full-length Flt-1 was preserved in these mice. We crossed sFlt-1$^{-/-}$ mice with ApoE$^{-/-}$ mice to generate sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice. For detail, please see Supplementary methods.

Heparin loading in mice

Male C57BL/6 mice (CLEA Japan, Tokyo, Japan) and sFlt-1$^{-/-}$ mice were intravenously injected with 10 IU/kg of heparin (Sigma-Aldrich, Saint Louis, MO, USA) or PBS via the tail vein, and blood samples were collected 10 minutes after injection by eye bleeding. Plasma levels of mouse sFlt-1, PlGF, and VEGF were measured with commercial sandwich ELISA kits (R&D systems, Minneapolis, MN, USA).

Organ culture of mouse thoracic aorta explants

C57BL/6 mice and sFlt-1$^{-/-}$ mice were sacrificed, and thoracic aortas were isolated. Aortic tissues were gently cut open, positioned so that the endothelial surface faced upward, and incubated for 24 hours with or without 10 IU/ml heparin. sFlt-1 levels in the collected medium were assayed.

All experiments were approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University.

Cells and culture experiments

HUAECs and HMVECs were purchased for in vitro assay (LONZA, Allendale, NJ, USA). For the purpose of investigating whether sFlt-1 is stored on endothelial cell surfaces, HMVECs were seeded on 6-well dishes at a cell density of 4 x 10^5 cells/well, and incubated with Medium 199 + endothelial growth medium-2, from which heparin was removed. Heparin was added to the medium in increasing amounts, and the conditioned medium and the cells were harvested for ELISA and RNA extraction, respectively, after 10 minutes of incubation.

In addition, HUAECs were seeded on 12-well dishes at a concentration of 2 x 10^5 cells/well and incubated with Dulbecco's modified Eagle's medium, and treated with serum from patients (CKD1 (control), CKD5, and CKD5D). After 48 hours of incubation, cells were harvested for RNA analysis and measurement of ROS (Image-iT LIVE Green Reactive Oxygen Species Detection Kit, Molecular Probes, Eugene, OR, USA). Expression levels of the ROS-related gene HO-1 were evaluated. Vitamin E was used to assess the effect of pharmaceutical inhibition on ROS-dependent pathways.

Measurement of atherosclerotic lesions

The Extent of atherosclerosis was assessed on thoracoabdominal aorta by staining for lipid deposition with oil-red-O and on aortic roots (Valsalva sinus) by staining with hematoxylin-eosine.

Expression of sFlt-1 mRNA

Expression levels of sFlt-1 mRNA were measured using SYBR green–based RT-PCR with gene-specific primers as follows:

Mouse	forward: 5′-AACAACAGAACCATGCACCA-3′
	reverse: 5′-AGGGCACTGGGCTTTCTTAT-3′
Human	forward: 5′-CCCTGCAACATTCAGGCACC-3′
	reverse: 5′-GAGCATCTCCTCCGAGCCTG-3′

First forward–reverse pair was designed to detect sFlt-1 in the mouse, while the second pair was designated to detect sFlt-1 in humans. RT-PCR was conducted using the ABI Prism Sequence Detection System 7700 (Applied Biosystems). For detail, see Supplementary methods.

Statistical analysis

Clinical data were as mean ± SD or median and interquartile range as appropriate, and experimental data were expressed as mean ± SEM.

Differences between two groups were determined by the Student's t test, Mann-Whitney's U test, or χ^2 test, and those between more than 2 groups were determined with one-way ANOVA or the Kruskal-Wallis test. The Bonferroni test was

available for post hoc multiple comparisons of the post-heparin PlGF/sFlt-1 ratio according to CKD stage. Spearman's rank-correlation coefficient was used to assess the correlation between two continuous variables. Cumulative incidence was estimated by the Kaplan-Meier method, and differences were assessed with the log-rank test. The associations between post-heparin PlGF/sFlt-1 ratios and the incidence of cardiovascular events were analyzed using parsimonious Cox regression models because of the small number of events. The final model was adjusted for seven covariates including age, sex, and cardiovascular risk factors. Two-sided $P<0.05$ was considered statistically significant. SPSS version 19.0 (SPSS Inc. IL, USA) was used to perform all statistical analyses.

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Titles and legend

Figure 1. Hypothesis representation of the balance between PlGF and sFlt-1.

In healthy subjects, the balance between PlGF and sFlt-1 is tightly maintained, but in CKD patients, this balance can be disrupted by down-regulation of sFlt-1, resulting in the progression of atherosclerosis and the promotion of plaque angiogenesis via the relative activation of atherogenic PlGF/Flt-1 signal.

Figure 2. Heparin loading test.

(a) Plasma sFlt-1 levels were dose-dependently increased by heparin injection (n=8).

(b) sFlt-1 levels were significantly lower in CKD patients (n=4) than in patients with normal renal function (n=4) at 5 minutes after heparin injection.

Figure 3. Plasma sFlt-1 and PlGF levels before and after heparin injection in CKD patients.

(a,b) Post-heparin sFlt-1 levels were positively correlated with eGFR in CKD patients (n=273), while pre-heparin sFlt-1 levels were negatively correlated with eGFR. (c-f) PlGF levels were positively correlated with eGFR, while low-dose heparin injection did not change PlGF or VEGF levels. (g) Post-heparin PlGF/sFlt-1 ratio was strongly correlated with eGFR (r=0.680). (h) The post-heparin PlGF/sFlt-1 ratio was significantly increased according to CKD stage, including patients receiving hemodialysis (n=343).

***P<0.001. Data are mean ± SD. r, correlation coefficient.

Figure 4. PlGF/sFlt-1 ratio and cardiovascular events.

(a) The pre-heparin PlGF/sFlt-1 ratio was not relevant to incidence of cardiovascular events (P=0.107). (b) The post-heparin PlGF/sFlt-1 ratio was a strong predictor of incidence of cardiovascular diseases (P=0.001).

Figure 5. sFlt-1 is released from endothelial cells by heparin administration. (a) Low-dose heparin (10 IU/kg) was administered via the tail veins of C57Bl/6 mice and sFlt-1$^{-/-}$ mice. Blood samples (0.7–1.0 ml) were drawn 10 minutes after heparin injection and sFlt-1 protein levels were analyzed by ELISA. n=8, $^{*}P$<0.05 vs. wild-type mice without heparin, $^{\#}P$<0.05 vs. sFlt-1$^{-/-}$ mice without heparin, respectively. (b) Thoracic aortas were excised and cut open for incubation with culture medium that included heparin (ex vivo). The medium was collected 24 hours after heparin treatment and sFlt-1 was measured by ELISA. n=8, $^{*}P$<0.05 vs. wild-type mice without heparin, $^{\#}P$<0.05 vs. sFlt-1$^{-/-}$ mice without heparin, respectively. (c) HMVECs were cultured with heparin for 10 minutes. The medium was collected 10 minutes after heparin treatment and sFlt-1 was measured by ELISA. n=6, $^{*}P$<0.05 vs. medium without heparin. (d) sFlt-1 mRNA was analyzed simultaneously in order to investigate sFlt-1 production in HMVECs. n=6, Data are mean ± SEM.

Figure 6. Serum from CKD patients induces endothelial damage and suppression of sFlt-1 production.

(a–c) HUAECs were cultured with serum from patients (Control, CKD5, CKD5D), and mRNA of sFlt-1, PlGF, and VEGF was measured by RT-PCR after 48 hours. n=8 (d–f) The markers of endothelial damage were also analyzed by RT-PCR. n=8, $^{*}P$<0.05 vs. CKD1, $^{\#}P$<0.05 vs. CKD5, respectively. Data are mean ± SEM.

Figure 7. Increased oxidative stress is associated with suppression of sFlt-1 production in endothelial cells.

(a) Levels of the oxidative stress marker HO-1 were increased in tissue rich in endothelial cells in 5/6 nephrectomized ApoE$^{-/-}$ mice. n=8, *P<0.05 vs. Control. (b) Intracellular ROS production was increased in HUAECs by cultivation with serum from CKD patients. n=8, *P<0.05 vs. Control. (c) Suppression of sFlt-1 mRNA production in HUAECs was rescued by vitamin E administration in the CKD5D group. n=4, *P<0.05 vs. CKD5D without vitamin E. (d) PlGF production in HUAECs was increased in the CKD5D group and vitamin E suppressed this elevation. n=4, *P<0.05 vs. CKD5D without vitamin E. (e–h) Expression levels of HO-1 and Nox-2 mRNA were increased in nephrectomized ApoE$^{-/-}$ mice compared to control ApoE$^{-/-}$ mice. n=6, Data are mean ± SEM.

Figure 8. sFlt-1$^{-/-}$ mice demonstrate increased atherosclerotic formation and macrophage infiltration.

sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice (a, c, e) and sFlt-1$^{-/-}$ ApoE$^{-/-}$ (b, d, f) mice were fed a high-cholesterol diet for 10 weeks and atherosclerotic lesions were compared between the two strains. Thoracoabdominal aortas were excised and stained with Oil Red O. Aortic roots were sectioned and stained with HE (magnification x5). Atherosclerotic lesions of the thoracoabdominal aorta (a, b, g) and aortic root (c, d, h) were exacerbated in sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice compared to sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice. (e, f, i) F4/80-positive macrophages (magnification x20) were increased in the plaques of aortic roots in sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice compared to sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice. n=8, *P<0.05

vs. sFlt-1$^{+/+}$ ApoE$^{-/-}$ mice. Data are mean ± SEM.

Figure 9. Hypothetical illustration for the localization and role of sFlt-1.

In the baseline condition of patients with normal renal function, the majority of sFlt-1 is stored on the endothelial cell surface, with a smaller amount in the circulation (a). In contrast, in the baseline condition of patients with renal dysfunction, a smaller amount of sFlt-1 is produced and stored on the endothelial cell surface, and a larger amount of sFlt-1 is in circulation (b). Heparin injection mobilizes membrane-associated sFlt-1 into circulation (c, d). Plasma levels of sFlt-1 after heparin injection are higher in the context of normal renal function than in renal dysfunction because smaller amounts of baseline sFlt-1 are stocked on the endothelial cell surface in renal dysfunction.

Table 1. Clinical Characteristics of Patients Stratified by CKD Stage

	Control and CKD patients (n=343)						
	Control (n=67)	3a (n=37)	3b (n=44)	4 (n=58)	5 (n=67)	5D (n=70)	*P*
Age, y	62 (33-75)	69 (60-76)	74 (67-78)	74 (66-79)	72 (60-77)	65 (59-72)	<0.001
Male sex, n (%)	35 (52)	22 (59)	26 (59)	31 (53)	44 (66)	46 (66)	0.442
Blood urea nitrogen, mg/dl	17 (14-21)	21 (17-25)	25 (22-32)	37 (30-52)	63 (44-78)	51 (40-65)	<0.001
Serum creatinine, mg/dl	0.7 (0.6-0.8)	1.0 (0.9-1.1)	1.3 (1.2-1.5)	2.1 (1.7-2.5)	5.1 (3.9-6.6)	7.8 (6.2-9.4)	<0.001
Proteinuria, n (%)	16 (24)	14 (38)	17 (39)	37 (63)	60 (90)	14 (93)	<0.001
Smoking, n (%)	23 (34)	19 (51)	23 (52)	30 (51)	41 (61)	48 (69)	0.002
Obesity, n (%)	11 (16)	5 (14)	13 (30)	18 (31)	7 (12)	9 (13)	0.02
Body mass index, kg/m^2	22.4 ± 3.8	23.1 ± 2.5	24.0 ± 3.6	24.1 ± 3.8	22.5 ± 3.3	22.2 ± 3.6	<0.001
Body weight, kg	55 (46-64)	57 (52-66)	58 (54-70)	60 (55-67)	55 (49-63)	58 (49-67)	0.21
Diabetes, n (%)	16 (24)	9 (24)	20 (45)	28 (47)	27 (40)	40 (57)	0.001
HbA1c, %	5.7 (5.4-6.3)	5.9 (5.3-6.2)	6.2 (5.6-7.2)	5.9 (5.6-6.7)	5.8 (5.5-6.1)	5.8 (5.3-6.4)	0.011

Hypertension, n (%)	24 (36)	22 (59)	31 (70)	49 (83)	59 (90)	66 (94)	<0.001
Dyslipidemia, n (%)	24 (36)	13 (35)	20 (45)	28 (47)	20 (30)	19 (27)	0.128
LDL cholesterol, mg/dl	98 (85-119)	114 (88-137)	115 (79-132)	92 (67-137)	88 (72-119)	95 (71-120)	0.036
Previous cardiovascular diseases, n (%)	18 (27)	9 (24)	8 (18)	19 (32)	9 (13)	28 (40)	0.011
Hemoglobin, g/dl	13.0 ± 1.9	12.6 ± 1.8	11.6 ± 2.1	10.0 ± 2.0	9.7 ± 1.2	10.4 ± 1.6	<0.001
CRP, mg/dl	3.9 ± 8.9	5.3 ± 8.9	4.6 ± 8.4	7.1 ± 11.4	7.4 ± 11.8	8.2 ± 11.7	0.149
BNP, pg/ml	23 (8-68)	39 (15-180)	56 (36-161)	125 (64-337)	243 (94-803)	264 (81-749)	<0.001
Pre-heparin sFlt-1, pg/ml	79 (59-103)	88 (64-134)	88 (66-111)	97 (75-122)	109 (88-137)	124 (106-159)	<0.001
Post-heparin sFlt-1, pg/ml	340 (257-490)	318 (245-431)	259 (222-352)	235 (178-284)	175 (140-237)	202 (176-240)	<0.001
Baseline PlGF, pg/ml	10.5 (6.9-14.0)	10.7 (7.2-13.9)	13.4 (10.6-16.2)	15.4 (10.2-19.1)	16.4 (12.6-20.1)	21.8 (17.9-28.1)	<0.001
Baseline VEGF, pg/ml	227 (110-458)	271 (137-435)	201 (144-383)	319 (149-498)	273 (156-361)	314 (185-539)	0.288
Pre-heparin PlGF/sFlt-1 ratio	0.116 (0.087-0.168)	0.109 (0.069-0.189)	0.153 (0.093-0.222)	0.141 (0.096-0.219)	0.142 (0.103-0.192)	0.183 (0.135-0.227)	<0.001

Post-heparin PlGF/sFlt-1 ratio	0.029 (0.018-0.045)	0.029 (0.021-0.049)	0.051 (0.036-0.073)	0.061 (0.039-0.101)	0.087 (0.059-0.125)	0.109 (0.084-0.133)	<0.001

Abbreviations: sFlt-1, soluble fms-like tyrosine kinase-1; PlGF, placental growth factor; VEGF, vascular endothelial growth factor; HbA1c, glycated hemoglobin; LDL, low-density lipoprotein; CRP, C-reactive protein; BNP, brain natriuretic peptide. Data are expressed as mean ± SD or as median (interquartile range), as appropriate.

Table 2. Multivariate Cox Regression Analysis of Variables Influencing the incidence of Cardiovascular Events

	Post-heparin PlGF/sFlt-1 ratio		
	Coefficient	Hazard ratio (95%CI)	*P*
Model 1	1.152	3.163 (1.564 to 6.399)	0.001
Model 2	1.113	3.043 (1.503 to 6.160)	0.002
Model 3	1.076	2.933 (1.395 to 6.168)	0.005
Model 4	1.047	2.849 (1.350 to 6.015)	0.006

Model 1: undjusted model. Model 2: adjusted age (10yr-difference), and sex. Model 3: Model 2 plus diabetes and hypertension. Model 4: Model 3 plus obesity, dyslipidemia, and smoking.

Table 3. mRNA Expression of sFlt-1, Flt-1, PlGF, and VEGF in Lung and Kidney in Control Mice and Nephrectomized ApoE$^{-/-}$ Mice

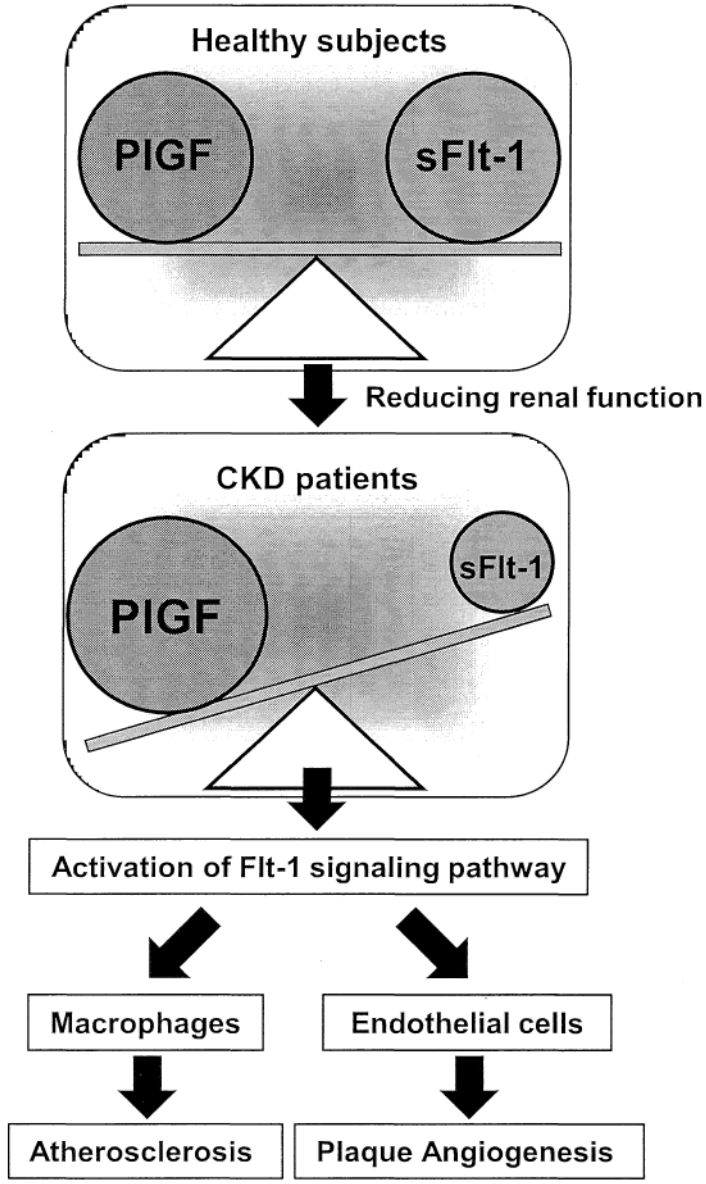
	Lung			Kidney		
mRNA/GAPDH	Control Mice	5/6 NR Mice	*P*	Control Mice	5/6 NR Mice	*P*
sFlt-1	6.02 ± 0.67	2.86 ± 0.32	0.0028	0.729 ± 0.032	0.408 ± 0.030	<0.001
PlGF	0.268 ± 0.024	0.510 ± 0.133	0.09	7.02 ± 0.44	9.78 ± 0.64	0.002
VEGF	0.422 ± 0.013	0.697 ± 0.067	<0.001	5.39 ± 0.30	8.00 ± 0.48	<0.001

5/6 NR Mice indicates 5/6 nephrectomized ApoE knockout mice. Data are expressed as mean ± SEM.

Table 4. Blood Biochemistry Data in sFlt-1$^{+/+}$ ApoE$^{-/-}$ Mice and sFlt-1$^{-/-}$ ApoE$^{-/-}$ Mice at the End of the Experiment

	sFlt-1$^{+/+}$ ApoE$^{-/-}$ (n=4)	sFlt-1$^{-/-}$ ApoE$^{-/-}$ (n=4)	*P*
Total cholesterol, mg/dl	2562.00 ± 150.41	2038.00 ± 128.88	0.056
LDL cholesterol, mg/dl	758.00 ± 53.00	541.67 ± 39.19	<0.05
HDL cholesterol, mg/dl	5.80 ± 0.58	6.67 ± 1.67	0.57
Triglycerides, mg/dl	30.00 ± 7.75	10.00 ± 5.57	0.12
Blood urea nitrogen, mg/dl	31.00 ± 1.67	32.60 ± 6.96	0.78
Serum creatinine, mg/dl	0.06 ± 0.02	0.07 ± 0.04	0.88

Abbreviations: LDL, low-density lipoprotein; HDL, high-density lipoprotein. Data are expressed as mean ± SEM.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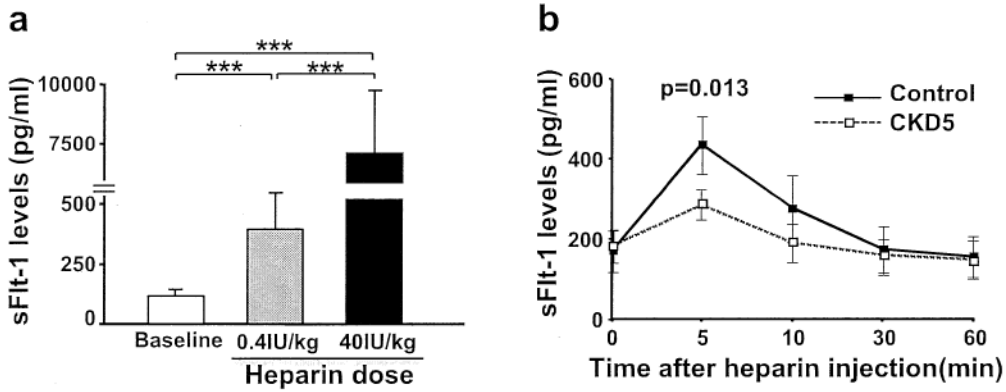
sFlt-1
Reducing renal function
CKD patients
PIGF
sFlt-1
Activation of Flt-1 signaling pathway
Macrophages
Endothelial cells
Atherosclerosis
Plaque Angiogenesis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sFlt-1 levels (pg/ml)
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500
250
0
Baseline
0.4IU/kg
40IU/kg
Heparin dose
b
p=0.013
Control
CKD5
sFlt-1 levels (pg/ml)
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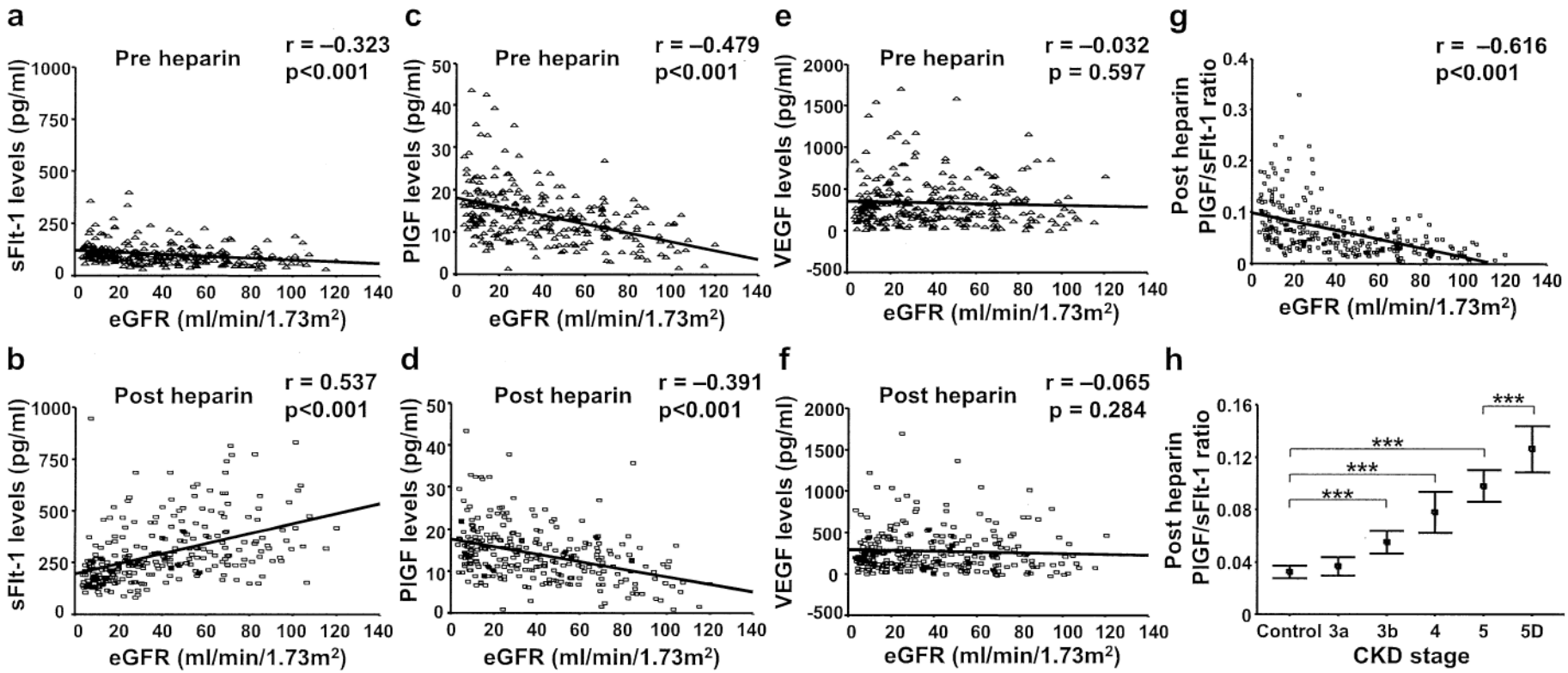
400
200
0
0
5
10
30
60
Time after heparin injection(min)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eGFR (ml/min/1.73m²)
b
Post heparin
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p<0.001
sFlt-1 levels (pg/ml)
eGFR (ml/min/1.73m²)
c
Pre heparin
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p<0.001
PlGF levels (pg/ml)
eGFR (ml/min/1.73m²)
d
Post heparin
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p<0.001
PlGF levels (pg/ml)
eGFR (ml/min/1.73m²)
e
Pre heparin
r = –0.032
p = 0.597
VEGF levels (pg/ml)
eGFR (ml/min/1.73m²)
f
Post heparin
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VEGF levels (pg/ml)
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g
r = –0.616
p<0.001
Post heparin PlGF/sFlt-1 ratio
eGFR (ml/min/1.73m²)
h
Post heparin PlGF/sFlt-1 ratio
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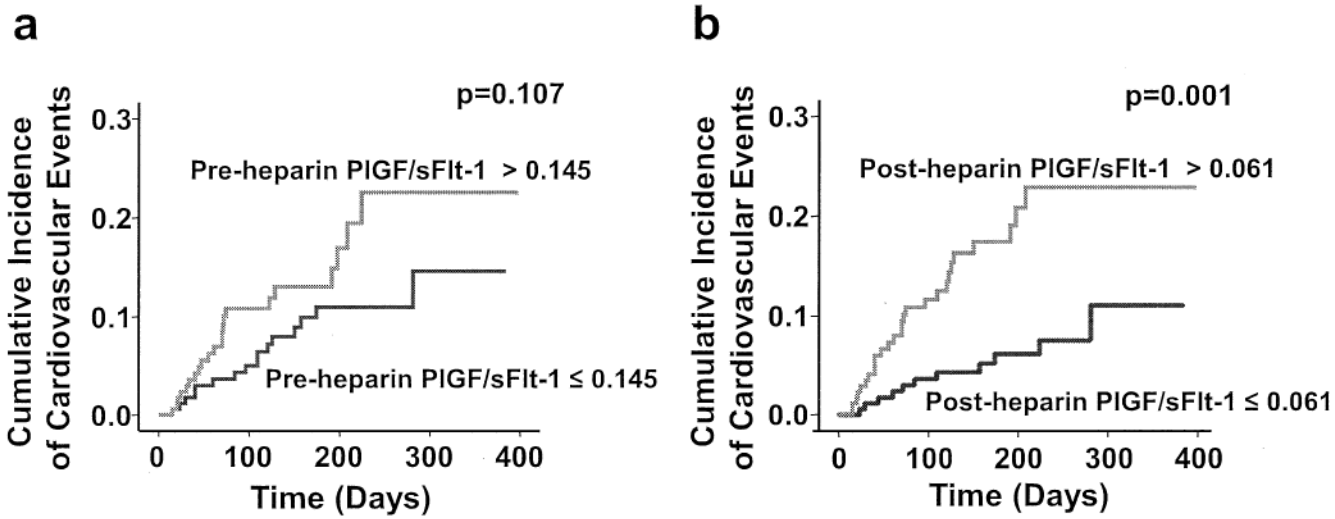
Control 3a 3b 4 5 5D
CKD stage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Pre-heparin PlGF/sFlt-1 ≤ 0.145
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0.2
0.1
0.0
0
100
200
300
400
Time (Days)
b
p=0.001
Post-heparin PlGF/sFlt-1 > 0.061
Post-heparin PlGF/sFlt-1 ≤ 0.061
Cumulative Incidence of Cardiovascular Events
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0.1
0.0
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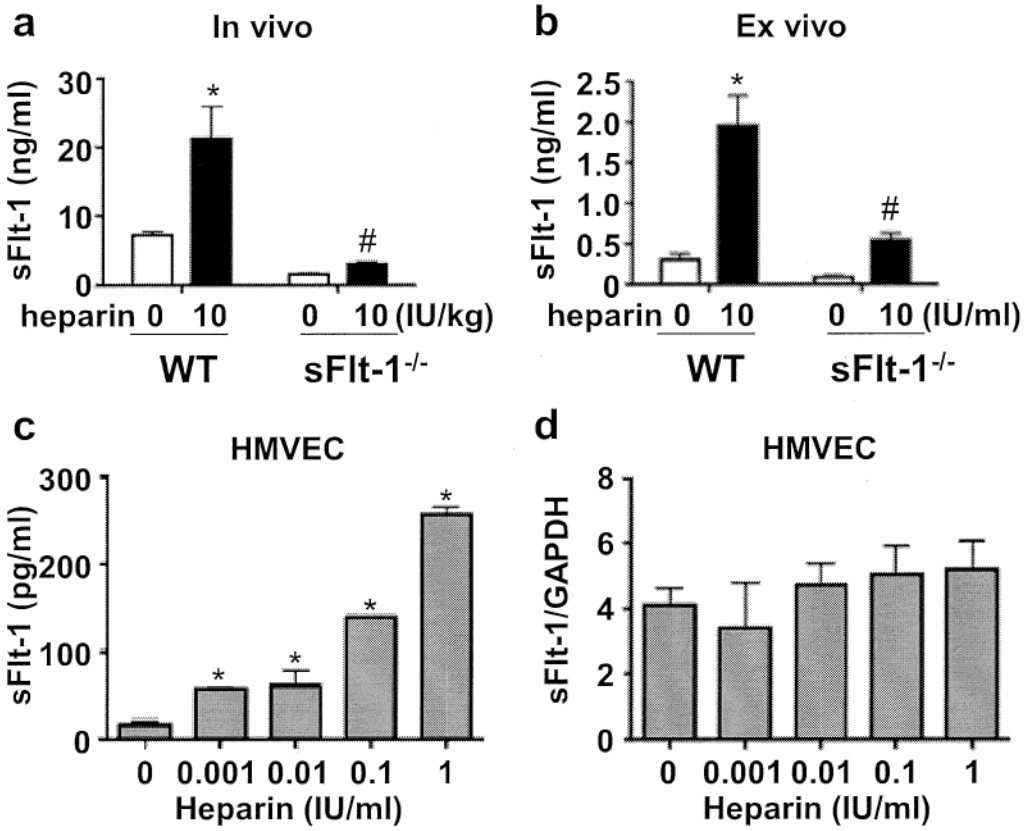
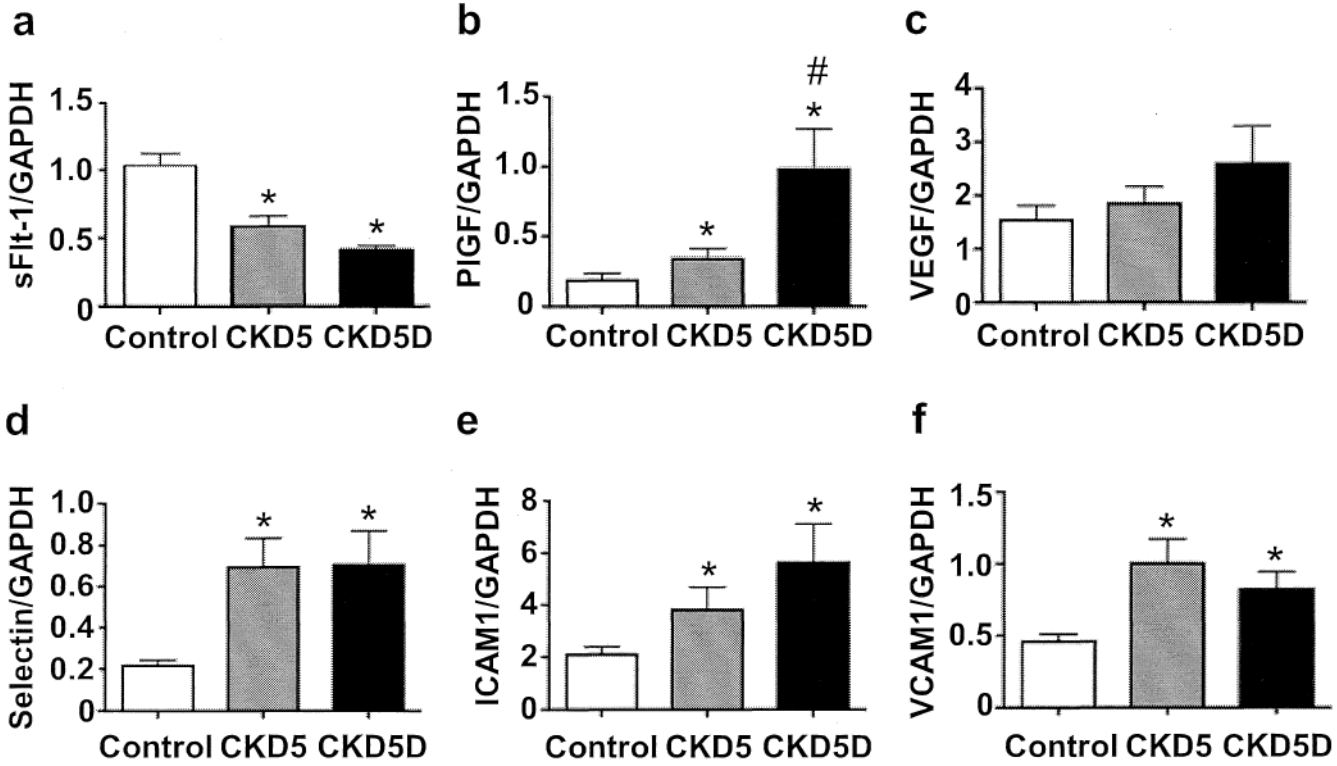
100
200
300
400
Time (Days)

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1.0
0.5
0
*
*
Control CKD5 CKD5D
b
PIGF/GAPDH
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1.0
0.5
0
#
*
*
Control CKD5 CKD5D
c
VEGF/GAPDH
4
3
2
1
0
Control CKD5 CKD5D
d
Selectin/GAPDH
1.0
0.8
0.6
0.4
0.2
0
*
*
Control CKD5 CKD5D
e
ICAM1/GAPDH
8
6
4
2
0
*
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VCAM1/GAPDH
1.5
1.0
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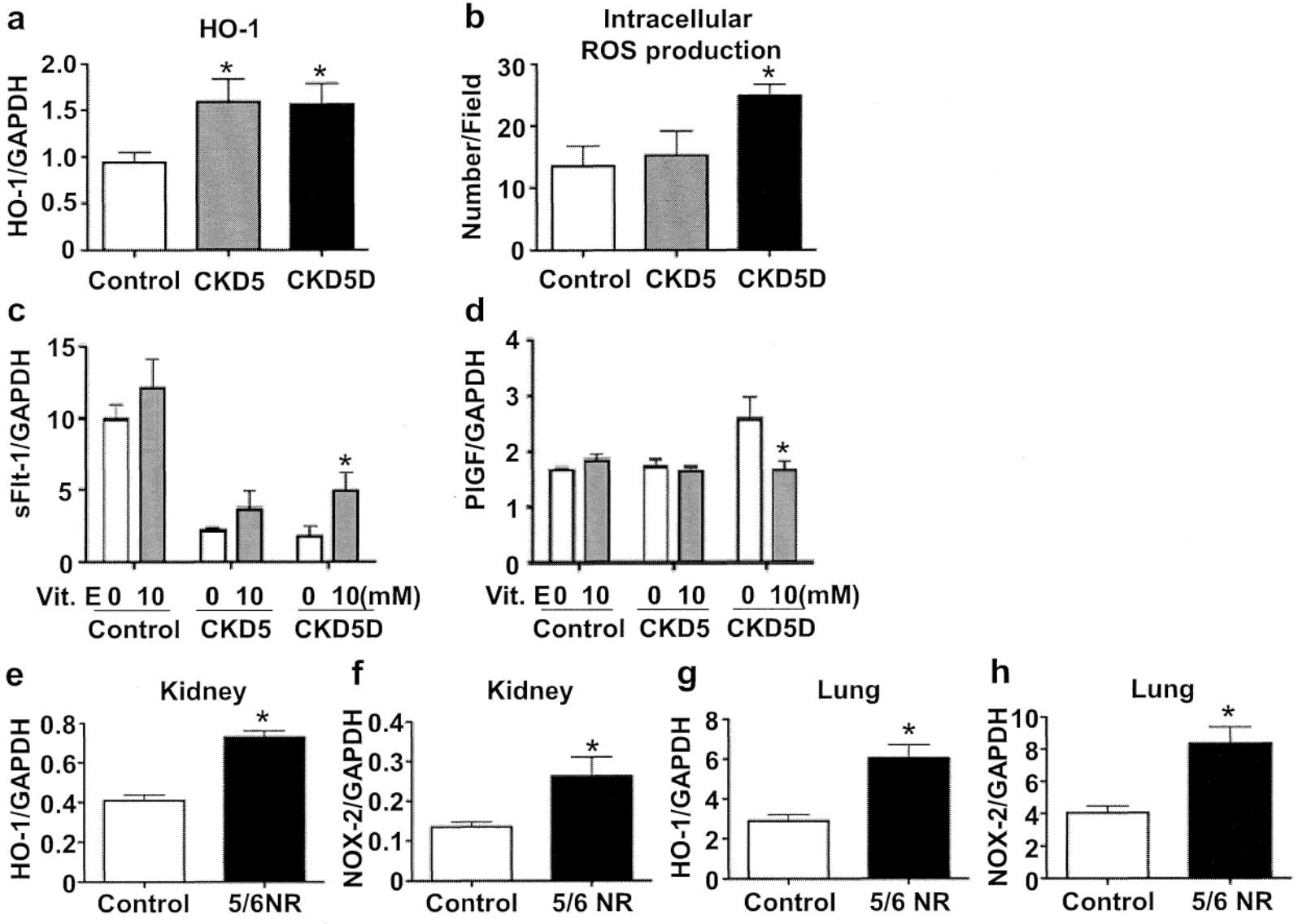
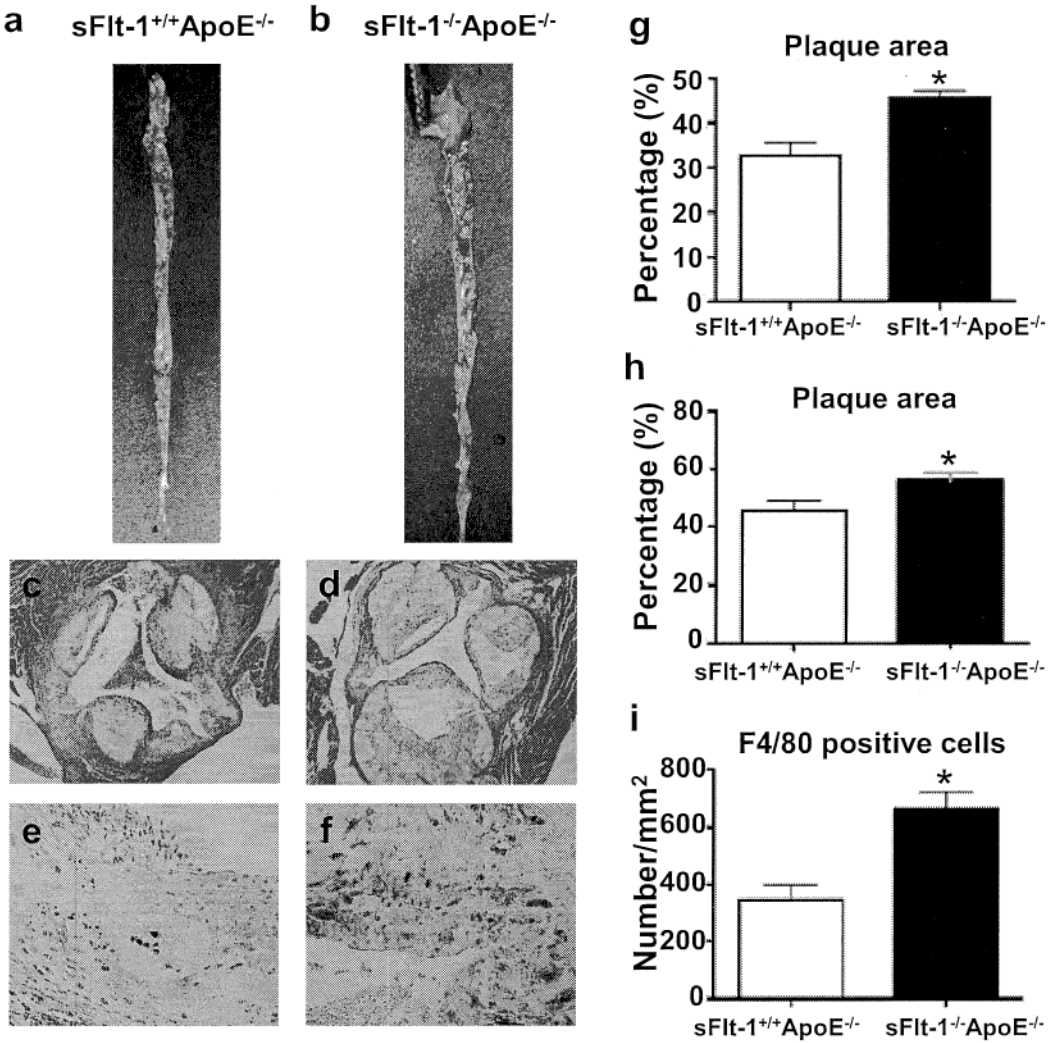
*
*
Control CKD5 CKD5D

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c
d
e
f
g
Plaque area
Percentage (%)
0
10
20
30
40
50
*
sFlt-1+/+ApoE-/-
sFlt-1-/-ApoE-/-
h
Plaque area
Percentage (%)
0
20
40
60
80
*
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sFlt-1-/-ApoE-/-
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F4/80 positive cells
Number/mm2
0
200
400
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800
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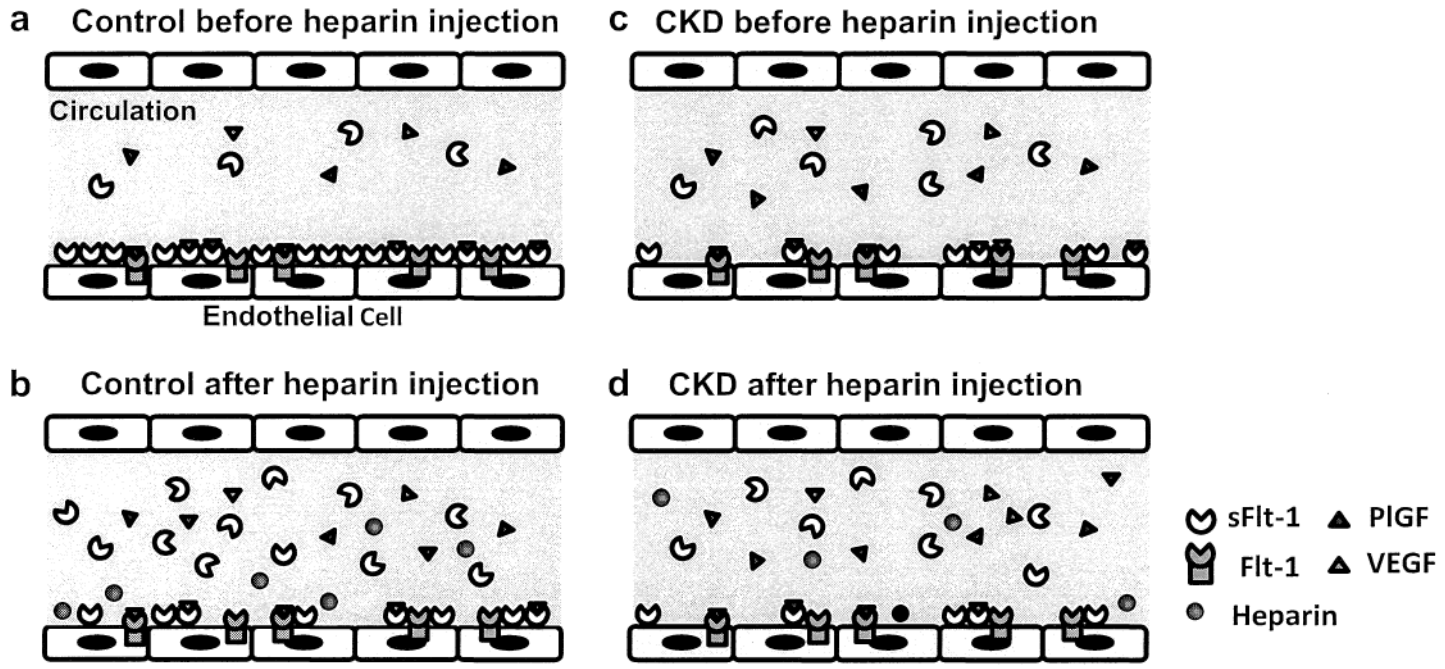
sFlt-1+/+ApoE-/-
sFlt-1-/-ApoE-/-


a Control before heparin injection
Circulation
Endothelial Cell
c CKD before heparin injection
b Control after heparin injection
d CKD after heparin injection
sFlt-1
PlGF
Flt-1
VEGF
Heparin

Suppressed Soluble Fms-Like Tyrosine Kinase-1 Production Aggravates Atherosclerosis in CKD : Evaluation by Circulating sFlt-1 Levels after Low-Dose Heparin Injection

Online supplement

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Contents

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Supplemental Methods

Clinical definition

We conducted patient interviews and performed laboratory tests to evaluate the following patient background characteristics: age, sex, body weight, body mass index, hypertension, diabetes, dyslipidemia, and past history of coronary artery disease (CAD). Hypertension was defined as systolic blood pressure ≥ 140 mmHg, diastolic blood pressure ≥ 90 mmHg, or current treatment with oral antihypertensive drugs. Diabetes was defined as fasting glucose ≥ 126 mg/dl or current treatment with oral hypoglycemic medications or insulin. Dyslipidemia was defined as low-density lipoprotein (LDL) cholesterol ≥ 140 mg/dl or current treatment with lipid-lowering medications. Previous CAD was defined by a history of myocardial infarction, angina pectoris, or coronary artery bypass grafting surgery. Patients were diagnosed with proteinuria if dipstick test results were "1+" or greater.

Estimated GFR (eGFR) was calculated using the Modification of Diet in Renal Disease equation revised for Japan: eGFR (ml/min/1.73 m^2) = 194 × (serum creatinine)$^{-1.094}$ × age$^{-0.287}$ (if female, ×0.739).[1] CKD stage was categorized according to a modified National Kidney Foundation classification as follows: Control, including Stages 1 and 2 (eGFR ≥ 60 ml/min/1.73 m^2); Stage 3a (eGFR 45–59 ml/min/1.73 m^2); Stage 3b (eGFR 30–44 ml/min/1.73 m^2); Stage 4 (eGFR 15–29 ml/min/1.73 m^2); Stage 5 (eGFR < 15 ml/min/1.73 m^2); and Stage 5D (hemodialysis patients). Control subjects consisted of patients visiting our department to undergo cardiac catheterization as well as healthy volunteers.

Cardiovascular events were defined as the composite of the following individual events occurring during the study period: fatal or non-fatal newly developed coronary artery disease, sudden cardiac death, peripheral arterial disease, congestive heart

failure requiring hospitalization, cerebrovascular disease, and aortic disease including rupture and dissection of aortic aneurysm.

Generation of sFlt-1$^{-/-}$ and sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice

sFlt-1 knockout mice were generated by the insertion of a targeting vector into the sFlt-1 genomic locus. The constructed targeting vector was introduced into embryonic stem (ES) cells on a C57BL/6 background for homologous recombination (Supplementary Figure 2A). Recombined ES cell clones were identified by PCR, and a successful single insertion was confirmed by the Southern blot analysis (Supplementary Figure 2B,C). Blastocysts containing ES cells with the floxed allele were implanted and chimeras were bred to obtain heterozygous mice that carry the targeted sFlt-1 locus in their germ line. The floxed PGK-neo cassette was removed by breeding with a CAG-Cre transgenic line. All breeding was done with mice under the C57BL/6 genetic background. Frequency of genotypes obtained from sFlt-1 Het–Het mating was shown in Supplementary Figure 2D. We crossed sFlt-1$^{-/-}$ mice with ApoE$^{-/-}$ mice to obtain sFlt-1$^{-/-}$ ApoE$^{-/-}$ mice.

RT–PCR

Quantitative real-time polymerase chain reaction (RT-PCR) was performed using commercially available or homemade primers and probes for the studied genes. mRNA was extracted from various frozen organ specimens, and cDNA was synthesized according to the standard protocol.

Expression levels of Flt-1 mRNA were measured using SYBR green real-time polymerase chain reaction with gene-specific primers as follows:

forward : 5′-CCCTGCAACATTCAGGCACC-3′

reverse : 5′-GGCTCGGGGACACCATTAGC-3′

The combination of forward and reverse was designated to detect Flt-1 in human. The levels of genes related to angiogenesis (PlGF, VEGF, Angiopoietin-2), oxidative stress (HO-1, a component of nonphagocyte-type NAHPD oxidase: NOX-2), and endothelial damage (Selectin, ICAM-1, VCAM-1) were detected using Taqman Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). Expression levels were normalized by use of glyceraldehyde-3-phosphate dehydrogenase as an endogenous control.

Expression of Flt-1 and sFlt-1 mRNA in vascular tissues

HUVECs (Human Umbilical Vein Endothelial Cells), CASMC (Normal Human Coronary Artery Smooth Muscle Cells) were purchased (LONZA; Allendale, NJ, USA) for the analysis of Flt-1 and sFlt-1 mRNA expressions. Monocytes were isolated from human blood taken from volunteer donors by Ficoll-Paque density gradient (Lymphocyte Separation Medium, MP Biomedicals; Irvine, CA, USA).

Macrophage preparation

To harvest peritoneal macrophages, the peritoneal cavity was washed with 5 ml of RPMI 10% FBS.[2] The pooled cells were then seeded in RPMI 10% FBS in 6-well

plates. After 6 hour of incubation at 37 ℃ in a moist atmosphere of 5% CO2 and 95% air, non-adhering cells on each plate were removed by rinsing with phosphate-buffered saline. The attached macrophages were grown in RPMI 10% for 12 hour, and then harvested for mRNA analysis.

Immunohistochemistry

Paraffin-embedded aortic roots were stained with antibodies against F4/80 (MCA497GA, 1:200; Serotec, Oxford, UK), and subsequently incubated with biotinylated secondary antibody (Mouse anti-Rat IgG: BD Pharmingen, San Diego, CA, USA), followed by amplification with the signal amplification system (CSA System, Dako; Carpinteria, CA, USA).

Isolated tissues of lung, heart, and muscle were embedded in paraffin, and were sectioned at the thickness of 6-μm with a cryostat as described previously.[3] Tissues were stained with antibodies against αSMA (1:200; Sigma-Aldrich, Saint Louis, MO, USA), and CD31 (1:200; BD Pharmingen, San Diego, CA ,USA). Vessel density was determined by counting the number of CD31-stained vessels and αSMA-stained vessels per high-power field (magnification, x200) in heart, lung, and muscle, respectively.

Supplementary information is available at Kidney International

Reference

1. Matsuo S, Imai E, Horio M, et al. Revised equations for estimated GFR from serum creatinine in Japan. Am J Kidney Dis 2009;53 982-992.

2. Takeda Y, Costa S, Delamarre E, Roncal C, Leite de Oliveira R, Squadrito ML, Finisguerra V, Deschoemaeker S, Bruyère F, Wenes M, Hamm A, Serneels J, Magat J, Bhattacharyya T, Anisimov A, Jordan BF, Alitalo K, Maxwell P, Gallez B, Zhuang ZW, Saito Y, Simons M, De Palma M, Mazzone M. Macrophage skewing by Phd2 haplodeficiency prevents ischaemia by inducing arteriogenesis. Nature 2011; 479:122-6.

3. Takeda Y, Uemura S, Iwama H, Imagawa K, Nishida T, Onoue K, Takemoto Y, Soeda T, Okayama S, Somekawa S, Ishigami K, Takaoka M, Kawata H, Kubo A, Horii M, Nakajima T, Saito Y. Treatment with recombinant placental growth factor (PlGF) enhances both angiogenesis and arteriogenesis and improves survival after myocardial infarction. Circ J 2009;73:1674-82.

Supplementary Figure Legends

Supplementary Figure 1. Expression levels of sFlt-1 and Flt-1 mRNA as determined by RT-PCR.

(a) Expression levels of sFlt-1 mRNA in lung and aorta were higher compared with other tissues. *n*=4. (b) Expression levels of Flt-1 and sFlt-1 mRNA in different types of cell were analyzed by Q-PCR. Both Flt-1 and sFlt-1 mRNA was expressed in HUVEC, CASMC, and Monocyte. *n*=3.

Supplementary Figure 2. Genotyping and survival of sFlt-1$^{-/-}$ mice

(a) The structures of the wild-type allele, targeting vector, targeting allele, and mutant allele are shown together with the relevant restriction sites. (b) Southern blot analysis of control (lane1) and sFlt-1 targeted (lane2) ES cells. HindIII and Acc65RI digestions of genomic DNA were used for Southern blot analysis with 5′ and 3′ probes, respectively. (c) PCR genotyping using yolk sac. WT, wild type; Het, heterozygous; KO, homozygous null; Positions of the 5' and 3' probes for Southern blot analysis and PCR genotyping primers (F1, R1 and R2) are shown in Supplementary Figure 2a. (d) Frequency of genotypes obtained from sFlt-1 Het-Het mating on a C57BL/6 genetic background.

Supplementary Figure 3. sFlt-1 gene expression and protein production in sFlt-1$^{-/-}$ mice.

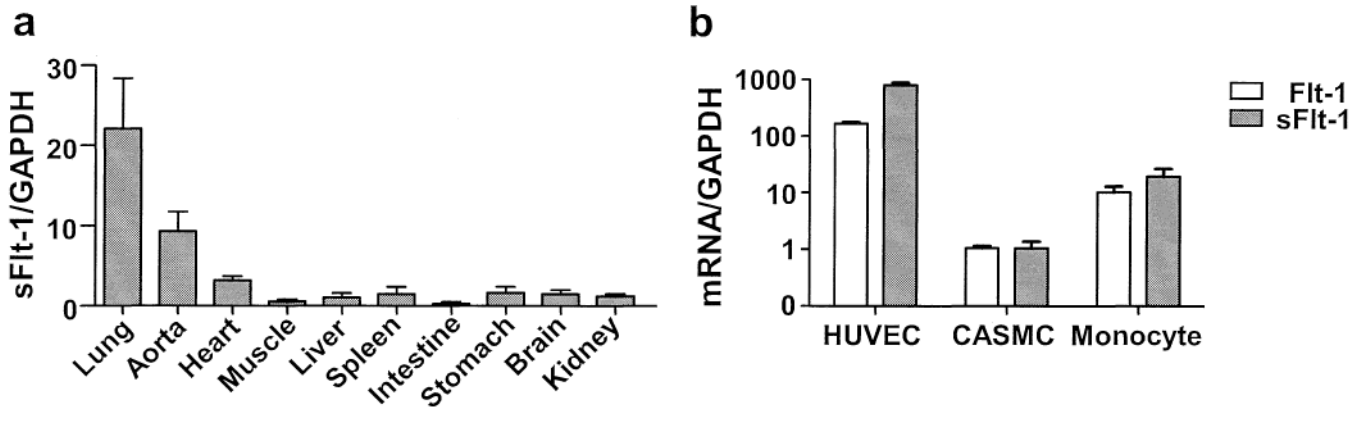
In wild-type mice and sFlt-1$^{-/-}$ mice, blood was drawn from the tail vain. Circulating levels of sFlt-1, PlGF, and VEGF were measured by ELISA and expression levels of sFlt-1, PlGF and VEGF mRNA were measured by RT-PCR. (a, b) The sFlt-1 gene was completely deleted in sFlt-1$^{-/-}$ mice, while circulating sFlt-1 levels in sFlt-1$^{-/-}$ mice (homozygous) were suppressed, with levels approximately half of those in wild-type mice. (c-f) mRNA expression levels and circulating levels of PlGF and VEGF in sFlt-1$^{-/-}$ mice were not different from those in wild-type mice. *n*=8, $^{*}P<0.05$ vs. wild-type mice, $^{\#}P<0.05$ vs. sFlt-1$^{+/-}$ mice, respectively. Data are mean ± SEM.

Supplementary Figure 4. Vessel Density in sFlt-1$^{-/-}$ mice.

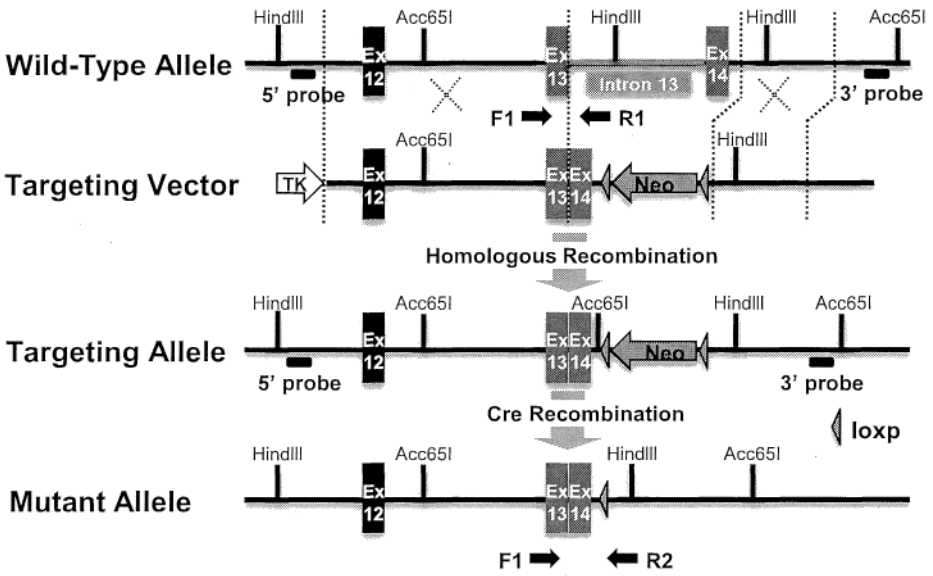
(a-f) Numbers of αSMA positive vessels and CD31 positive vessels in sFlt-1$^{-/-}$ mice were not significantly different from those in wild-type mice in heart, lung, and muscle, respectively (magnification, x200).

Supplementary Figure 5. Post-heparin sFlt-1 levels and cardiovascular diseases.
Of 343 subjects, 68 patients underwent cardiac catheterization and 55 of these were newly diagnosed with coronary artery disease. (a, b) Pre-heparin sFlt-1 levels were not associated with severity of coronary artery stenosis, whereas post-heparin sFlt-1 levels showed significant association (P=0.07, P=0.033, respectively). (c, d) Kaplan-Meier curves of the cumulative incidence of cardiovascular diseases for pre-heparin sFlt-1 levels and post-heparin sFlt-1 levels. There was no difference in the incidence of cardiovascular events between patients with higher and lower pre-heparin sFlt-1 levels (P=0.103). The incidence of cardiovascular events in patients with higher post-heparin sFlt-1 levels tended to be higher than in patients with lower post-heparin sFlt-1 levels (P=0.06).

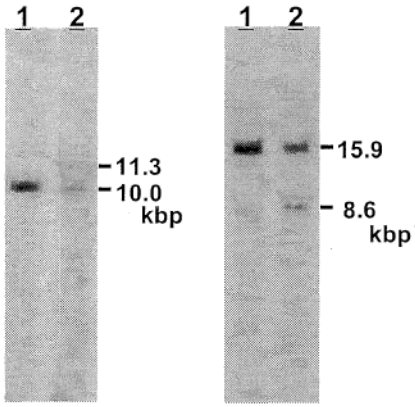
Supplementary Figure 6. Macrophage is a target cell for PlGF/Flt-1 signal transduction.
(a, b) Expression levels of TNFα and MCP-1 mRNA in peritoneal macrophages. n=6, *P<0.05 vs. wild-type mice, Data are mean ± SEM


a
sFlt-1/GAPDH
30
20
10
0
Lung
Aorta
Heart
Muscle
Liver
Spleen
Intestine
Stomach
Brain
Kidney
b
mRNA/GAPDH
1000
100
10
1
0
HUVEC
CASMC
Monocyte
Flt-1
sFlt-1

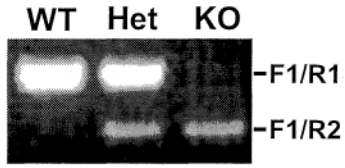
a



b

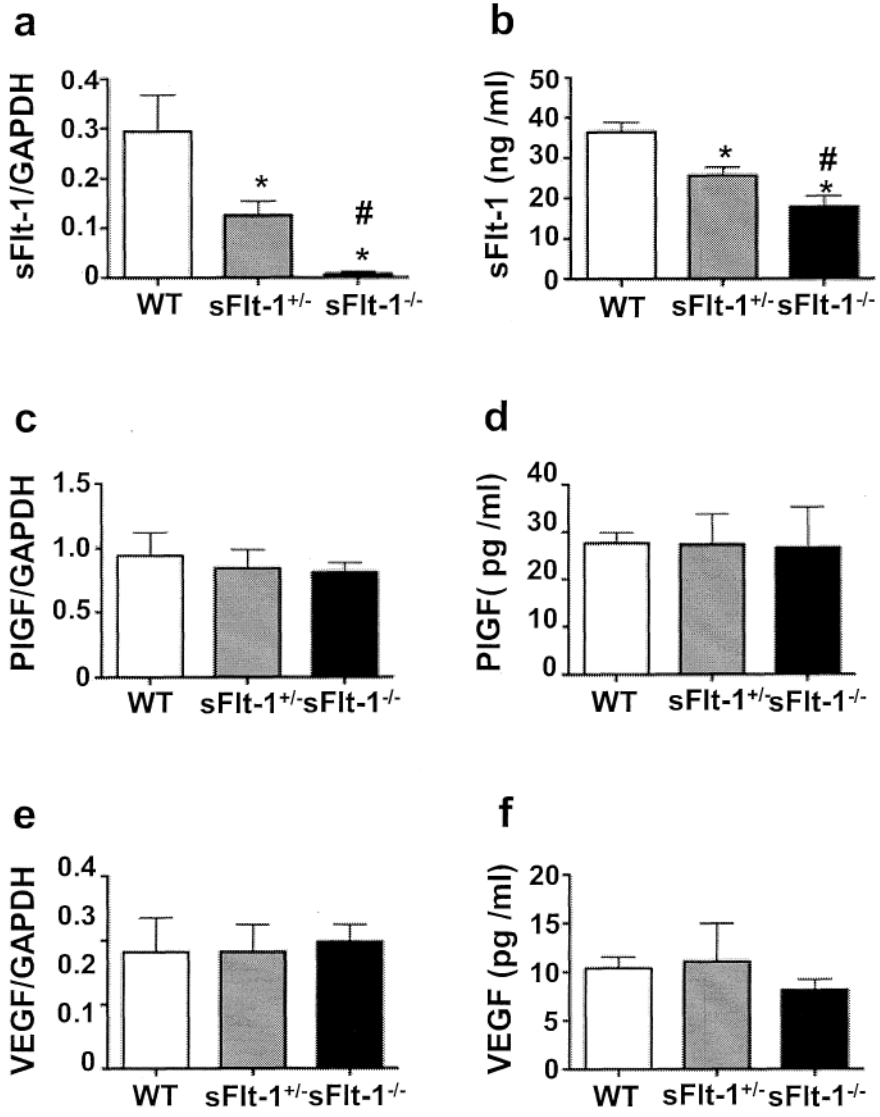


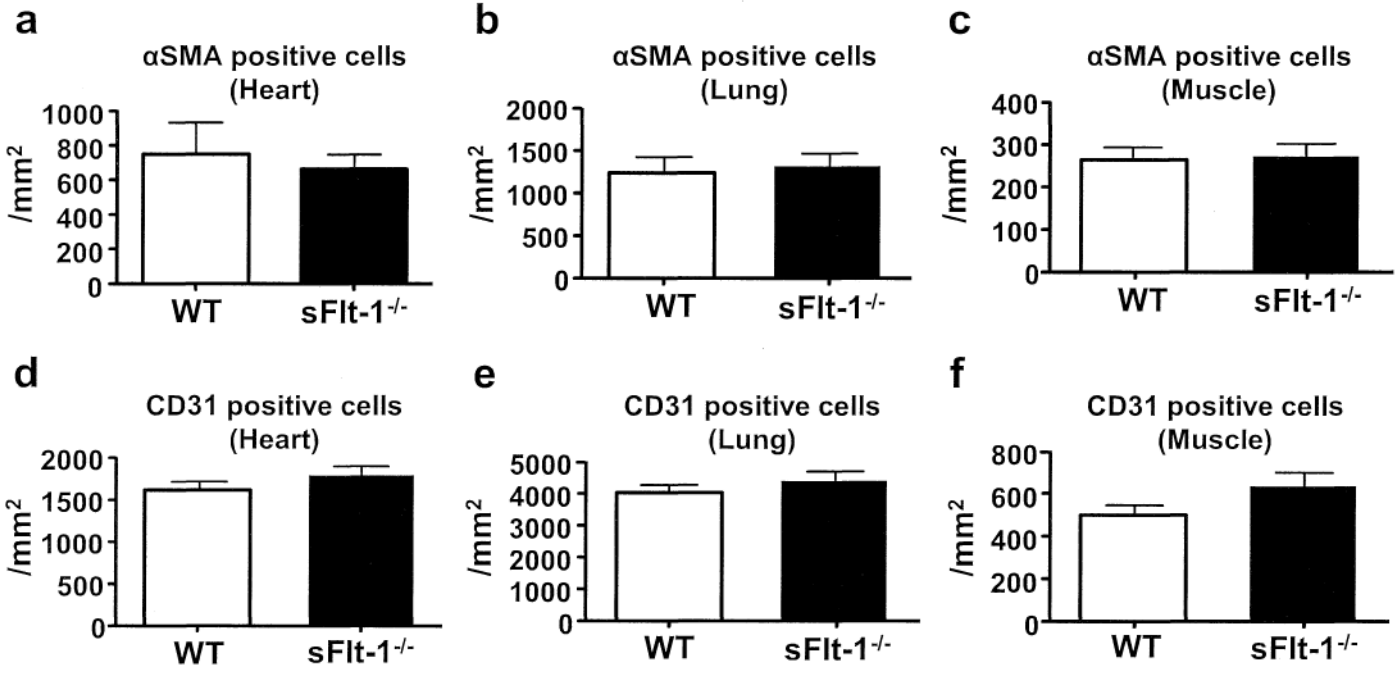
c

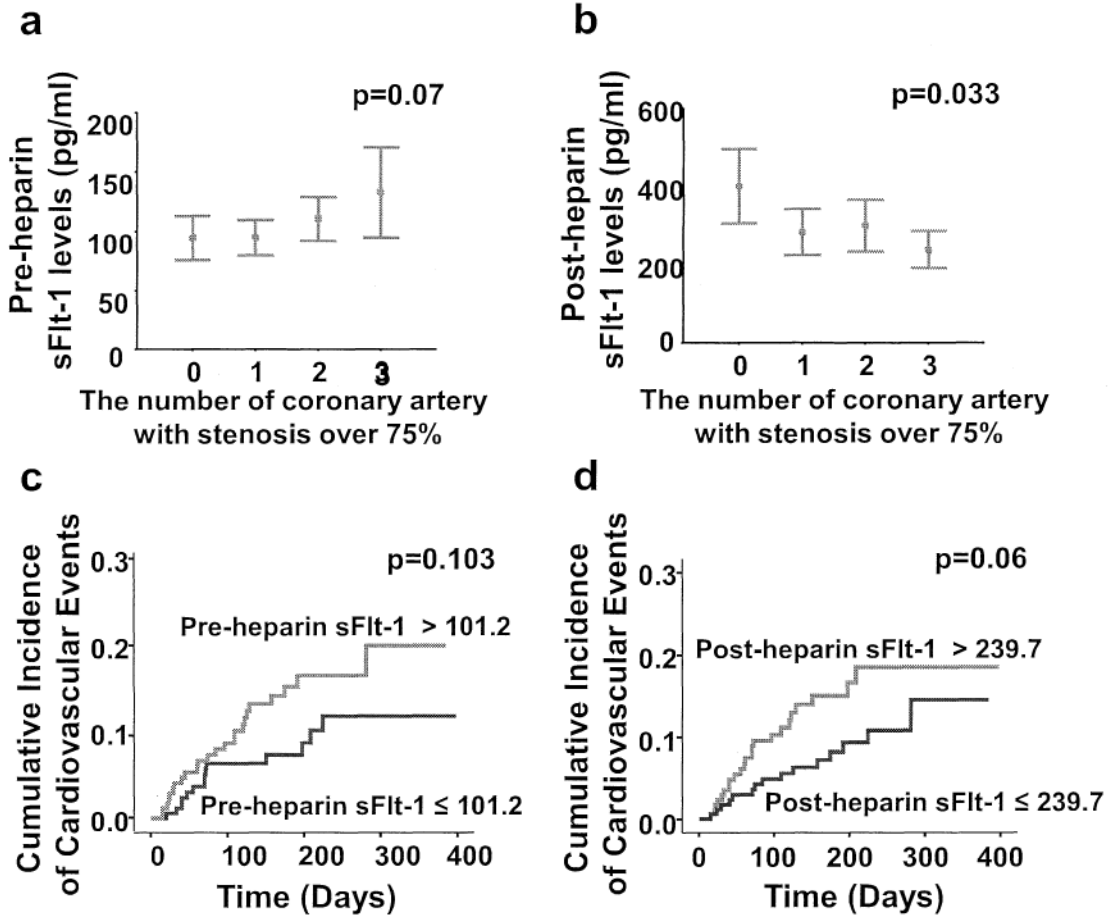


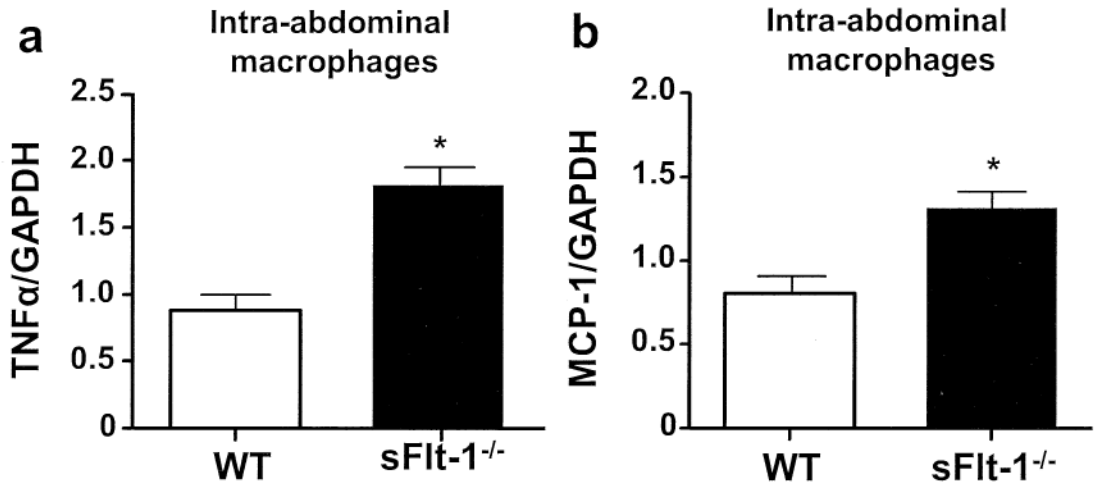
d

Total numbers	WT	Het	KO
414	125 (30.2%)	218 (52.7%)	71 (17.1%)




a
αSMA positive cells
(Heart)
/mm2
1000
800
600
400
200
0
WT
sFlt-1-/-
b
αSMA positive cells
(Lung)
/mm2
2000
1500
1000
500
0
WT
sFlt-1-/-
c
αSMA positive cells
(Muscle)
/mm2
400
300
200
100
0
WT
sFlt-1-/-
d
CD31 positive cells
(Heart)
/mm2
2000
1500
1000
500
0
WT
sFlt-1-/-
e
CD31 positive cells
(Lung)
/mm2
5000
4000
3000
2000
1000
0
WT
sFlt-1-/-
f
CD31 positive cells
(Muscle)
/mm2
800
600
400
200
0
WT
sFlt-1-/-


a
p=0.07
Pre-heparin sFlt-1 levels (pg/ml)
200
150
100
50
0
0 1 2 3
The number of coronary artery with stenosis over 75%
b
p=0.033
Post-heparin sFlt-1 levels (pg/ml)
600
400
200
0
0 1 2 3
The number of coronary artery with stenosis over 75%
c
p=0.103
Cumulative Incidence of Cardiovascular Events
0.3
0.2
0.1
0.0
Pre-heparin sFlt-1 > 101.2
Pre-heparin sFlt-1 ≤ 101.2
0 100 200 300 400
Time (Days)
d
p=0.06
Cumulative Incidence of Cardiovascular Events
0.3
0.2
0.1
0.0
Post-heparin sFlt-1 > 239.7
Post-heparin sFlt-1 ≤ 239.7
0 100 200 300 400
Time (Days)


a
Intra-abdominal macrophages
TNFα/GAPDH
2.5
2.0
1.5
1.0
0.5
0
*
WT
sFlt-1-/-
b
Intra-abdominal macrophages
MCP-1/GAPDH
2.0
1.5
1.0
0.5
0
*
WT
sFlt-1-/-