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Reduction of Circulating Soluble Fms-Like Tyrosine Kinase-1 Plays a Significant Role in Renal Dysfunction Associated Aggravation of Atherosclerosis

Kenji Onoue, Shiro Uemura, Yukiji Takeda, Satoshi Somekawa, Hajime Iwama, Keiichi Imagawa, Taku Nishida, Yoshinobu Morikawa, Yasuhiro Takemoto, Osamu Asai, Tsunenari Soeda, Satoshi Okayama, Kenichi Ishigami, Kimihiko Nakatani, Hiroyuki Kawata, Manabu Horii, Tamio Nakajima, Yasuhiro Akai, Masayuki Iwano and Yoshihiko Saito

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Reduction of Circulating Soluble Fms-Like Tyrosine Kinase-1 Plays a Significant Role in Renal Dysfunction–Associated Aggravation of Atherosclerosis

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Background—Renal dysfunction is commonly accompanied by a worsening of atherosclerosis; however, the underlying molecular mechanism is not fully understood. We examined the role played by soluble fms-like tyrosine kinase-1 (sFlt-1), an endogenous antagonist of the proatherogenic cytokine placental growth factor (PlGF), in the worsening of atherosclerosis in patients with renal dysfunction and in an animal model of renal failure.

Methods and Results—In this study, 329 patients who received cardiac catheterization and 76 patients who underwent renal biopsy were enrolled. Both plasma sFlt-1 levels and renal sFlt-1 mRNA expression were positively correlated with estimated glomerular filtration rate ($P<0.01$). The PlGF/sFlt-1 ratio was negatively correlated with estimated glomerular filtration rate ($P<0.01$), whereas plasma PlGF levels were not affected by it. The PlGF/sFlt-1 ratio was significantly higher in patients with multivessel coronary artery disease than in patients with single-vessel or no coronary artery disease. The reduction of circulating sFlt-1 and renal sFlt-1 mRNA levels was confirmed in five-sixths (5/6)–nephrectomized apolipoprotein E–deficient mice that developed experimental renal dysfunction. Atherosclerotic plaque area and macrophage infiltration into the plaque were significantly higher in 5/6–nephrectomized apolipoprotein E–deficient mice than in control mice, but replacement therapy with recombinant sFlt-1 significantly reduced both plaque formation and macrophage infiltration.

Conclusions—The present study demonstrates that a reduction in the circulating levels of sFlt-1 is associated with the worsening of atherosclerosis that accompanies renal dysfunction. (***Circulation.* 2009;120:2470-2477.**)

Key Words: atherosclerosis ■ coronary disease ■ growth substances ■ heart failure ■ kidney

Chronic kidney disease is a worldwide public health problem not only because it leads to end-stage renal failure[1–3] but also because it is an independent risk factor for atherosclerosis-related cardiovascular events.[4,5] Accumulating evidence indicates that atherosclerosis is often worsened in patients with renal dysfunction,[6–9] and the risk of cardiovascular disease increases sharply as the estimated glomerular filtration rate (eGFR) declines.[10] Additionally, more than 50% of deaths among end-stage renal failure patients are due to cardiovascular events.[11] Although it is clear that most cardiovascular events associated with renal dysfunction result from atherosclerosis, the underlying molecular mechanism responsible for the worsening of atherosclerosis in chronic kidney disease is not yet fully understood. Consequently, an effective therapeutic strategy is still lacking.

Clinical Perspective on p 2477

Fms-like tyrosine kinase 1 (Flt-1), which is a receptor tyrosine kinase and a member of the vascular endothelial growth factor (VEGF) receptor family,[12] is a specific receptor for placental growth factor (PlGF) and VEGF-A. Soluble Flt-1 (sFlt-1), which consists of the 6 extracellular immunoglobulin-like domains of Flt-1, circulates as an endogenous antagonist of both PlGF and VEGF-A. PlGF is thought to exacerbate atherosclerosis by enhancing angiogenesis and the migration of monocytes/macrophages into the arterial wall.[13] Consistent with that idea, administration of an antibody against Flt-1 exerts an antiatherogenic effect in atherosclerosis-prone apolipoprotein E–deficient (apoE-deficient) mice by inhibiting the early growth of atherosclerosis

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rotic plaques and infiltration by macrophages.[14] Therefore, to further understand the pathological significance of the PlGF–Flt-1 system in renal dysfunction–related atherosclerosis, we measured plasma levels of sFlt-1 and PlGF in patients with renal dysfunction. Here we show that circulating sFlt-1 levels are reduced in patients with renal dysfunction in proportion to disease severity, whereas there is no change in plasma PlGF levels. Moreover, renal production of sFlt-1 is diminished in patients with renal dysfunction. In a mouse model, replacement treatment with recombinant human sFlt-1 reduced worsening of atherosclerosis induced by five-sixths (5/6) nephrectomy.

Methods

Clinical Study

Patient Population

In the present study, 329 consecutive patients admitted to Nara Medical University Hospital to undergo diagnostic cardiac catheterization for angina pectoris or congestive heart failure or to undergo follow-up coronary angiography after myocardial infarction were enrolled for evaluation of the relationship among renal function, plasma sFlt-1 levels, and the severity of coronary artery disease. In addition, 76 patients admitted to the hospital to undergo renal biopsy to diagnose the cause of proteinuria or renal dysfunction were enrolled for investigation of renal expression of sFlt-1. The clinical parameters assessed included age, sex, coronary risk factors, body weight, and serum creatinine. We calculated eGFR using a modified Modification of Diet in Renal Disease equation: eGFR ($mL \cdot min^{-1} \cdot 1.73\ m^{-2}$) = $194 \times (\text{serum creatinine})^{-1.094} \times age^{-0.287} \times 0.739$ (if female).[15] Clinical study protocols were approved by our institutional ethics committee (No. 2002-009, Nara Medical University Ethics Committee), and written informed consent was obtained in all cases from either the patient or his/her family members.

Cardiac Catheterization and Blood Sampling

Patients were recruited from February 2005 to March 2007. Those with acute coronary syndrome, evidence of malignant disease, or an unwillingness to participate were excluded. On the basis of the findings of coronary angiography and left ventriculography, a diagnosis was made, and the severity of coronary atherosclerosis was evaluated in terms of the number of vessels with >75% stenosis or Gensini's scoring method[16] (see the Methods section of the online-only Data Supplement for details). The severity of coronary artery disease was assessed by 2 independent angiographers who were blinded to the patients' backgrounds. Patients with dilated cardiomyopathy, hypertrophic cardiomyopathy, hypertensive heart disease, and valvular heart disease were categorized as having congestive heart failure. Patients with vasospastic angina pectoris or chest pain syndrome were categorized as "other."

At the beginning of the cardiac catheterizations, we collected blood samples before the injection of heparin. Samples were collected from the aortas of all enrolled patients, and samples from the coronary sinus, the hepatic vein, and the renal vein were collected simultaneously in 14 patients. Subsequently, plasma or serum samples were respectively added to EDTA anticoagulant tubes or plain tubes with serum separating agent and stored at −80°C until assayed. We measured plasma levels of sFlt-1 and PlGF and serum levels of VEGF using commercially available ELISA kits (DVR100B, DPG00, and DVE00, respectively; R&D Systems, Minneapolis, Minn; see the Methods section of the online-only Data Supplement for details).

Renal Biopsy and mRNA Analysis

Seventy-six consecutive patients who underwent diagnostic renal biopsy between May 2002 and October 2007 were enrolled. Patients with evidence of malignant disease or who were unwilling to participate were excluded. Renal specimens were stored at −80°C until assayed. We extracted mRNA from the specimens and generated cDNA as described previously.[17] Relative sFlt-1 mRNA levels were then determined by quantitative real-time polymerase chain reaction with cDNA samples with primers 5′-CCCTGCAAGATTCAGGCACC-3′ (forward) and 5′-GAGCATCTCCTCCGAGCCTG-3′ (reverse), which correspond to a unique sequence in the human sFlt-1 mRNA. Levels of sFlt-1 mRNA were normalized to those of GAPDH mRNA (see the Methods section of the online-only Data Supplement for details).

Experimental Study

We performed an experimental study to confirm the relationship between renal dysfunction and cardiovascular disease observed in the clinical study.

Animals

Male apoE-deficient mice (C57BL/6 background) were purchased from Taconic Farms (Hudson, NY) and maintained in a temperature-controlled room with a 12-hour light/dark cycle and free access to water and standard chow until they were 11 weeks old. Thereafter, they were maintained on a Western diet (16.5% fat, 1.25% cholesterol, 0.5% sodium cholate) until they were 22 weeks old. All experiments were approved by the Ethics Review Committee for Animal Experimentation of Nara Medical University.

Experimental Renal Failure and Injection of Recombinant Human sFlt-1 Replacement Therapy

At 8 weeks of age, mice were randomly assigned to a 5/6 nephrectomy (chronic renal failure; removal of one kidney and two thirds of the other kidney) or a control group. The 5/6 nephrectomy operation was performed as described previously.[18–20] Recombinant human sFlt-1 (rhsFlt-1, amino acids 1 to 338) was made from human Flt1 [sFlt-1 (D1–3)], which contains 3 immunoglobulin-like domains in its N-terminal region. The accuracy of production was confirmed in vitro and in vivo before the replacement therapy. Details can be found in the online-only Data Supplement. We administered rhsFlt-1 intraperitoneally at a dose of 15 ng per gram of body weight 3 times per week for 10 weeks beginning when the mice were 12 weeks old. Control mice received phosphate-buffered saline (PBS) at the same intervals over the same period. The mice were euthanized at 22 weeks under general anesthesia with pentobarbital sodium, after which blood samples were collected from the right ventricle, and the serum was stored at −80°C until analyzed. The hearts, aortas, and kidneys were excised and stored likewise.

Blood and mRNA Analysis

Serum urea, creatinine, total cholesterol, and triglyceride levels were assayed with enzymatic kits (Wako Pure Chemical Industries, Osaka, Japan). We also measured mouse sFlt-1, mouse PlGF-2, mouse VEGF, and human sFlt-1 using ELISAs (MVR100, MP200, MMV00, and DVR100B, respectively; R&D Systems). We extracted mRNA from frozen renal specimens and synthesized cDNA using standard protocols. Relative levels of mouse sFlt-1 mRNA were then determined by real-time polymerase chain reaction with cDNA samples with primers 5′-CTCTAGAAGACTCGGGCACC-3′ (forward) and 5′-GAGCGTTTCCTCTGGGCCTG-3′ (reverse), which corresponds to a unique sequence of mouse sFlt-1 mRNA. Levels of sFlt-1 mRNA were normalized to those of GAPDH mRNA.

Measurement of Atherosclerotic Lesions and Histological Examination

To quantify atherosclerotic plaque formation, atherosclerotic lesions within the thoracoabdominal aorta were stained with oil red O, and thin slices of aortic root (Valsalva sinus) were stained with Masson's trichrome. The aortic root slices were also labeled with anti-monocyte/macrophage antibody (MOMA-2; ABR Affinity Bioreagents, Golden, Colo). Plaque areas and MOMA-2–stained areas were traced by 2 independent examiners who were blinded to the specimens' background and were measured with ImageJ version 1.41 software (http://rsb.info.nih.gov/ij/) as described previously.[21]

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Table 1. Clinical Characteristics of the Patients

Variables	Cardiac Catheterization and Blood Analysis			Renal Biopsy and mRNA Analysis		
	eGFR ≥60 mL · min^{-1} · 1.73 m^{-2} (n=207)	eGFR <60 mL · min^{-1} · 1.73 m^{-2} (n=122)	Total (n=329)	eGFR ≥60 mL · min^{-1} · 1.73 m^{-2} (n=50)	eGFR <60 mL · min^{-1} · 1.73 m^{-2} (n=26)	Total (n=76)
Male, n (%)	153 (74)	86 (71)	239 (73)	31 (62)	17 (65)	48 (63)
Age, y (mean±SD)	64±10	69±10‡	66±10	48±16	56±12*	51±15
Risk factors, n (%)						
Hypertension	112 (54)	96 (79)‡	208 (63)	15 (30)	15 (58)*	30 (39)
Dyslipidemia	117 (57)	51 (42)†	168 (51)	9 (18)	13 (50)†	22 (29)
Diabetes	74 (36)	45 (37)	119 (36)	16 (32)	13 (50)	29 (38)
Smoking	130 (63)	76 (62)	206 (63)	4 (8)	8 (31)†	12 (16)
Obesity	82 (40)	33 (27)*	115 (35)	7 (14)	7 (27)	14 (18)
Diagnosis, n (%)						
Congestive heart failure	29 (14)	17 (14)	46 (14)	...	...	...
Angina pectoris	44 (21)	40 (33)*	84 (26)	...	...	...
Old myocardial infarction	88 (43)	45 (37)	133 (40)	...	...	...
Other	46 (22)	20 (16)	66 (20)	...	...	...
Diabetic nephropathy	...	...	...	14 (28)	13 (50)	27 (36)
IgA glomerular nephritis	...	...	...	18 (36)	9 (35)	27 (36)
Minimal-change nephrotic syndrome	...	...	...	8 (16)	2 (8)	10 (13)
Minor change	...	...	...	10 (20)	2 (8)	12 (16)

*P<0.05, †P<0.01, ‡P<0.001 vs eGFR ≥60 mL · min^{-1} · 1.73 m^{-2}.

Statistical Analysis

Continuous data are expressed as mean±SEM unless otherwise indicated. The significance of differences between 2 groups was determined with the Student t test and that between more than 3 groups was determined with 1-way ANOVA. Post hoc pairwise comparisons were performed with the Tukey-Kramer test in the clinical study and the Bonferroni/Dunn test in the experimental study. To assess correlations between 2 continuous variables, Pearson's correlation coefficient analysis and simple linear regression were performed. Multiple linear regression was performed to determine the variables that affected the sFlt-1 value. Values of P<0.05 were considered statistically significant. All statistics were calculated with Stat View for Windows, version 5.0 (SAS Institute Inc, Cary, NC).

Results

Clinical Study

Patient Characteristics

The clinical characteristics of the patients receiving cardiac catheterization and blood analysis are shown in Table 1. Among the patients, 27 were receiving maintenance hemodialysis. The characteristics of the patients enrolled in the renal biopsy study are also shown in Table 1. This study included no patients receiving hemodialysis.

Plasma sFlt-1 Levels

We analyzed sFlt-1 levels in plasma obtained from the aorta. A significant positive correlation was found between sFlt-1 levels in plasma from the aorta and eGFR (r=0.32, P<0.001; Figure 1A). Multiple linear regression revealed the sFlt-1 levels were not influenced by age, sex, or coronary risk factors but were affected only by eGFR (Table 2). To determine the site of sFlt-1 production, we measured sFlt-1 levels in plasma collected from the aorta, coronary sinus, hepatic vein, and renal vein of 14 patients. Among these 4 vessels, sFlt-1 levels were significantly higher in the renal vein than in any of the others (aorta, coronary sinus, hepatic vein, and renal vein: 325.6±43.4, 336.6±39.8, 172.3±67.8,

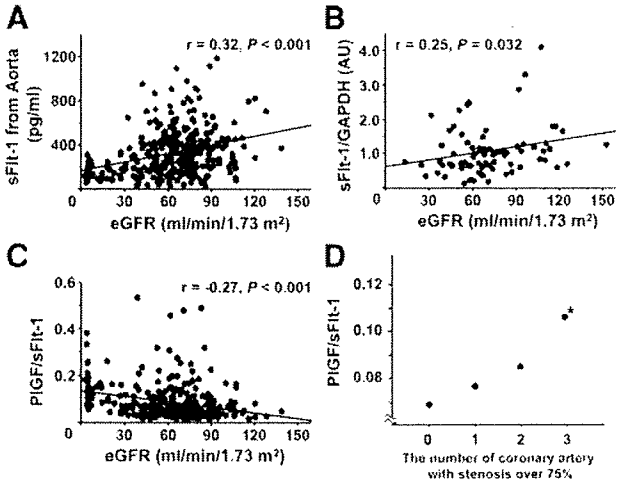

Figure 1. Plasma sFlt-1 and PlGF levels and expression of sFlt-1 mRNA. A, A significantly positive correlation was found between plasma levels of sFlt-1 from aorta and eGFR. n=329; r=0.32; P<0.001. B, A significantly positive correlation was found between relative levels of sFlt-1 mRNA in human renal biopsy specimens and eGFR. n=76; r=0.25; P=0.032. C and D. Relationship between plasma sFlt-1 and PlGF levels and coronary atherosclerosis. PlGF/sFlt-1 ratios plotted against eGFR (C) and extent of coronary atherosclerosis (D). A significantly negative correlation was found between PlGF/sFlt-1 ratio and eGFR (r=−0.27; P<0.001). Furthermore, PlGF/sFlt-1 ratio was significantly different according to the number of coronary arteries that showed >75% stenosis. Ninety-five percent confidence interval is also presented in A–C. *P<0.05 vs patients without coronary artery stenosis. Data are mean±SEM in D.

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Table 2. Multiple Linear Regression to Assess Variables Affecting the Value of sFlt-1

Variables	Coefficient	95% Confidence Interval	P
eGFR	2.85	1.96–3.74	<0.001
Age	1.51	−0.82 to 3.84	0.204
Male sex	−23.2	−84.3 to 38.0	0.456
Hypertension	11.0	−37.2 to 59.1	0.655
Dyslipidemia	4.39	−41.0 to 49.8	0.849
Diabetes	8.12	−38.0 to 54.2	0.729
Smoking	−13.3	−68.4 to 41.9	0.636
Obesity	8.86	−38.5 to 56.2	0.713

If a patient was male or had the disease or the habit, then 1 was given as the variable; if not, then 0 was given as the variable.

and 692.5±115.8 pg/mL, respectively; $P<0.001$; online-only Data Supplement Figure IA), which indicates that the kidney is a possible source of circulating sFlt-1. Moreover, there was a strong correlation between sFlt-1 levels in plasma from the aorta and plasma from the renal vein (n=126, $r=0.70$, $P<0.001$; online-only Data Supplement Figure IB).

Specific Expression Profile of sFlt-1 mRNA in Human Renal Biopsy Samples

To confirm renal production of sFlt-1 and assess the effect of renal dysfunction, we developed a specific quantitative real-time polymerase chain reaction system to measure the levels of sFlt-1 mRNA in human renal biopsy specimens (online-only Data Supplement Figures IIIA and IIIB). Notably, not only was sFlt-1 mRNA present in the biopsied samples, but its level also had a significantly positive correlation with eGFR ($r=0.25$, $P=0.032$; Figure 1B).

Coronary Artery Disease Worsens With Progression of Renal Dysfunction

The number of coronary arteries with >75% stenosis was significantly different according to renal dysfunction ($P=0.007$; online-only Data Supplement Figure IC). Scores obtained by Gensini's method for evaluating global coronary atherosclerosis were also significantly different according to renal dysfunction ($P=0.015$; online-only Data Supplement Figure ID).

Relationship Between PlGF/sFlt-1 Ratio and Coronary Artery Disease

Levels of sFlt-1 in plasma collected from the aorta tended to be lower ($P=0.0656$; online-only Data Supplement Figure IE), and there was a corresponding tendency for PlGF to be higher as the number of diseased vessels increased ($P=0.0513$; online-only Data Supplement Figure IF). In contrast, plasma PlGF levels were unaffected by differences in renal function (online-only Data Supplement Figure IIA); consequently, the PlGF/sFlt-1 ratio had a significantly negative correlation with eGFR ($r=-0.27$, $P<0.001$; Figure 1C). Although plasma levels of sFlt-1 and PlGF did not differ significantly, the PlGF/sFlt-1 ratio was significantly different according to the number of coronary arteries that showed >75% stenosis (PlGF/sFlt-1 ratio in patients with 0-, 1-, 2-, and 3-vessel disease: 0.07±0.01, 0.08±0.01, 0.09±0.01, and 0.11±0.01, respectively; $P=0.023$; Figure 1D).

Experimental Study

Effects of 5/6 Nephrectomy on Physical and Biochemical Parameters in ApoE-Deficient Mice

The 5/6 nephrectomy procedure had no significant effects on body weight, blood pressure, heart rate, or lung/body weight ratio compared with control apoE-deficient mice (Table 3). In contrast, heart/body weight ratios at the end of the study were significantly higher in 5/6-nephrectomized mice than in control mice, and serum urea and creatinine levels were also significantly higher. Total serum cholesterol and triglyceride levels were also higher in the 5/6-nephrectomized mice.

Serum sFlt-1 concentration was lower in the 5/6-nephrectomized mice (11.45±0.44 versus 13.24±0.39 ng/mL, $P<0.001$; Figure 2A), whereas serum PlGF-2 concentration was higher in those mice than in control mice (20.4±3.8 versus 10.2±2.1 pg/mL, $P<0.01$; Figure 2C), which made the PlGF-2/sFlt-1 ratio significantly higher in 5/6-nephrectomized mice than in control mice (0.86±0.22 to

Table 3. Hemodynamics and Biological Parameters in Control and 5/6-Nephrectomized ApoE-Deficient Mice at the End of the Study

	Control Mice		5/6-Nephrectomized Mice	
Characteristics	PBS (n=10)	sFlt-1 (n=10)	PBS (n=11)	sFlt-1 (n=12)
Body weight, g	26.0±0.6	26.0±0.5	25.2±0.5	25.6±0.4
Systolic blood pressure, mm Hg	87±2.7	88±3.4	97±5.4	97±2.6
Diastolic blood pressure, mm Hg	50±2.1	52±1.4	55±3.7	58±3.2
Heart rate, bpm	658±16	650±27	595±21	641±17
Heart/body weight ratio (mg/g)	4.05±0.07	4.18±0.09	4.71±0.07‡	4.53±0.09‡
Lung/body weight ratio (mg/g)	5.38±0.07	5.41±0.12	5.54±0.12	5.42±0.11
Blood urea nitrogen, mg/dL	28.6±1.2	35.1±4.1	67.0±4.4‡	67.4±3.5‡
Creatinine, mg/dL	0.70±0.08	1.12±0.13	1.30±0.09†	1.63±0.19‡
Total cholesterol, mg/dL	2242±122	2176±147	3018±164†	2797±181*
Triglyceride, mg/dL	116.7±8.5	160.9±29.6	243.4±44.8*	240.1±60.9

Data are mean±SEM.
*$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs control PBS.

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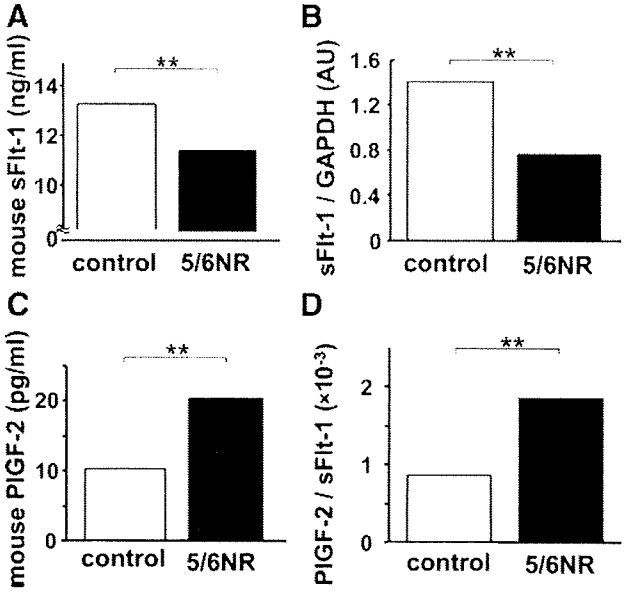

Figure 2. The effect of 5/6 nephrectomy (5/6NR) on serum sFlt-1 and PlGF-2 levels and renal expression of sFlt-1 mRNA in apoE-deficient mice. A and B, Both serum sFlt-1 levels (A) and renal sFlt-1 mRNA expression (B) were significantly lower in 5/6-nephrectomized apoE-deficient mice than in control apoE-deficient mice. C, Serum PlGF-2 levels were significantly higher in 5/6-nephrectomized apoE-deficient mice than in control mice. D, PlGF-2/sFlt-1 ratios were also higher in 5/6-nephrectomized mice than in control mice. $^{**}P<0.01$. Data are mean±SEM.

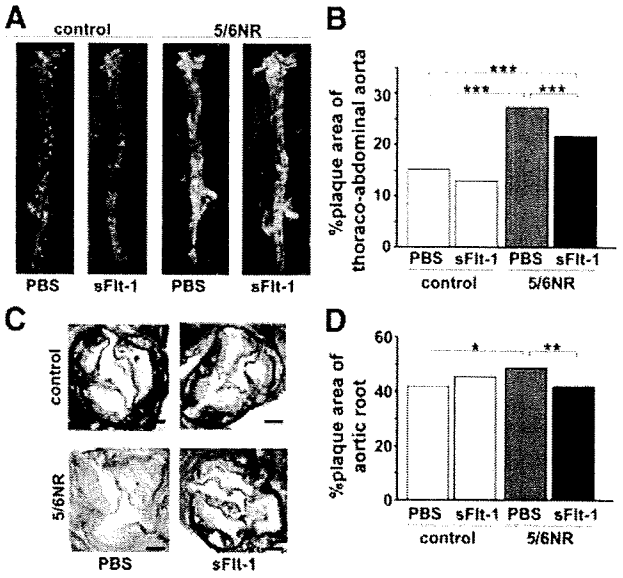

Figure 3. Atherosclerotic plaque formation in thoracoabdominal aorta and aortic root in apoE-deficient mice. Plaque formation in the thoracoabdominal aorta (A and B) and aortic root (C and D) was exacerbated by concomitant renal dysfunction and was mitigated to some extent by repeated intraperitoneal injection of rhsFlt-1. Repeated injections of rhsFlt-1 to control apoE-deficient mice had no significant effect on plaque formation. $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$. Data are mean±SEM. Bar=200 μm. 5/6NR indicates 5/6 nephrectomy.

1.86±0.38 $\times 10^{-3}$; $P<0.01$; Figure 2D). Renal expression of sFlt-1 mRNA was significantly lower in 5/6-nephrectomized mice (1.38±0.13 versus 0.73±0.11 arbitrary units normalized to GAPDH mRNA; $P<0.01$; Figure 2B). Serum VEGF concentrations were similar in 5/6-nephrectomized and control apoE-deficient mice (118.2±4.7 versus 138.0±7.7 pg/mL, $P=0.062$).

5/6 Nephrectomy Aggravates Atherosclerosis and Macrophage Infiltration in ApoE-Deficient Mice

As shown in Figure 3, the relative plaque areas in the thoracoabdominal aorta and aortic root were significantly larger in 5/6-nephrectomized apoE-deficient mice treated with PBS than in control apoE-deficient mice (thoracoabdominal aorta: 27.3±1.1% versus 15.2±0.9%, $P<0.001$; aortic root: 48.8±2.0% versus 41.9±1.4%, $P=0.014$). Moreover, Figure 4 shows that there was significantly greater macrophage infiltration into the atherosclerotic plaque of the aortic root in 5/6-nephrectomized mice than in control mice (33.3±1.8% versus 21.4±1.6%, $P<0.001$).

Effect of rhsFlt-1 Administration in ApoE-Deficient Mice

Repetitive intraperitoneal administrations of rhsFlt-1 had no effect on hemodynamics or biochemical parameters in apoE-deficient mice, as summarized in Table 3. Endogenous renal expression of sFlt-1 mRNA did not change in either the 5/6-nephrectomy or the control group.

Administration of rhsFlt-1 to control apoE-deficient mice had no significant effect on atherosclerotic plaque area compared with mice administered PBS (thoracoabdominal aorta: 13.0±0.8% versus 15.2±2.4%, $P=0.187$; aortic root: 45.7±2.0% versus 41.9±1.4%, $P=0.185$). However, replacement treatment with repeated injections of rhsFlt-1 to 5/6-nephrectomized apoE-deficient mice reduced the progression of atherosclerosis compared with 5/6-nephrectomized mice administered PBS (thoracoabdominal aorta: 21.6±1.4% versus 27.3±1.1%, $P<0.001$; aortic root: 41.8±2.0% versus 48.8±2.0%, $P<0.01$; Figure 3). In 5/6-nephrectomized mice, repeated rhsFlt-1 administration significantly reduced macrophage infiltration into atherosclerotic plaques compared with controls (28.4±2.1% versus 33.3±1.8%, $P=0.024$; Figure 4), although in control apoE-deficient mice, macrophage infiltration into atherosclerotic plaques did not differ between mice administered rhsFlt-1 and those administered PBS (17.8±1.7% versus 21.4±1.6%, $P=0.205$).

Discussion

A worsening of atherosclerosis commonly accompanies renal dysfunction, but the underlying molecular mechanism is not yet fully understood. The present study demonstrates that renal production of sFlt-1 and the corresponding plasma levels of the peptide decline with progression of renal dysfunction in both clinical and experimental settings. Furthermore, administration of rhsFlt-1 inhibits renal dysfunction–induced exacerbation of atherosclerosis in an apoE-deficient mouse model. Given that sFlt-1 is an endogenous antagonist of PlGF, a proatherogenic factor, the present findings suggest that the decline of circulating sFlt-1 in patients with renal dysfunction may play a significant role in the worsening of their atherosclerosis.

sFlt-1 contains the extracellular ligand-binding domain of the full-length, membrane-spanning Flt-1 receptor and is generated by alternative splicing of the same pre-mRNA that

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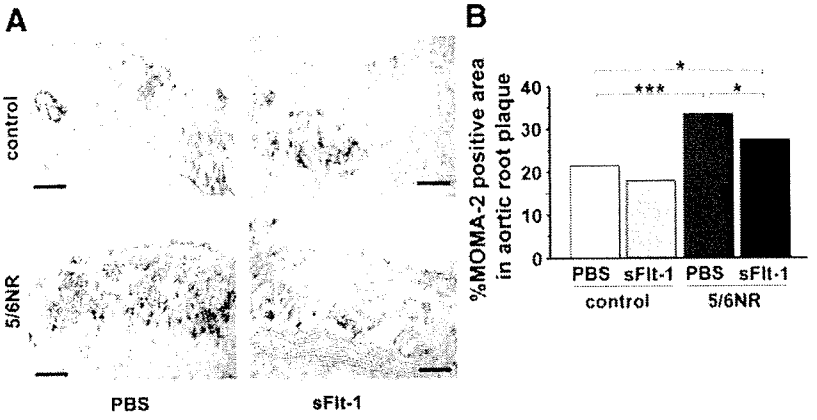

Figure 4. Infiltration of macrophages into atherosclerotic plaque in aortic roots from apoE-deficient mice. Infiltrating macrophages were stained with MOMA-2 (A). Infiltration was enhanced by progression of renal dysfunction and was reduced somewhat by repeated intraperitoneal injection of rhsFlt-1 (A and B). $^{*}P<0.05$; $^{***}P<0.001$. Data are mean±SEM. Bar=40 μm. 5/6NR indicates 5/6 nephrectomy.

encodes Flt-1.[22] Flt-1 mRNA is reportedly expressed in vascular endothelial cells in the lung, heart, kidney, and brain,[23] as well as in placental trophoblasts,[24] monocytes/macrophages,[25] and renal mesangial cells, especially in patients with mesangial proliferative glomerulonephritis.[26] However, studies of the expression profile of sFlt-1 mRNA under normal and pathophysiological conditions are lacking. In the present study, we confirmed that sFlt-1 mRNA is expressed in the kidney. Furthermore, we showed that there is a significant step-up in plasma sFlt-1 levels between the renal vein and aorta and that plasma sFlt-1 levels in the renal vein correlate significantly with those in the aorta. This suggests that renal sFlt-1 production makes a considerable contribution to the levels of peripheral circulating sFlt-1. In patients with renal dysfunction, both renal sFlt-1 production and levels of circulating sFlt-1 decline with progression of renal dysfunction. Moreover, these reductions in renal expression of sFlt-1 mRNA and circulating sFlt-1 were confirmed in a mouse model of chronic renal dysfunction.

To interpret the effects of reduced plasma levels of sFlt-1 in renal dysfunction, it is necessary to consider the relationship between sFlt-1 and its ligand, PlGF. Recent studies have shown that in addition to its angiogenic effects,[27] PlGF exerts such proatherogenic effects as recruitment and adhesion of monocytes, production of proteolytic factors, induction of thrombus formation by stimulation of tissue factor secretion, and plaque destabilization.[13,25,28–30] Because sFlt-1 acts as a natural PlGF antagonist,[12] we presume that when the circulating sFlt-1/PlGF ratio is reduced, the action of PlGF is augmented. In the peripheral circulation, free PlGF, free sFlt-1, and the PlGF–sFlt-1 complex are present simultaneously. Although it is not yet clear which of these most closely reflects PlGF–Flt-1–mediated proatherogenic activity, and it is not clear how the assay system for PlGF or sFlt-1 cross-reacts with the PlGF–sFlt-1 complex, the present study suggests that the PlGF/sFlt-1 ratio is most closely correlated with the severity of atherosclerosis in both patients with renal dysfunction and 5/6-nephrectomized apoE-deficient mice. Although further study is needed to elucidate the clinical significance of the PlGF/sFlt-1 ratio, we hypothesized that reduced plasma sFlt-1 levels are at least associated with a relative increase in PlGF–Flt-1–mediated proatherogenic signaling in renal dysfunction.

To test this idea, we investigated whether exogenous administration of rhsFlt-1 would reverse the exacerbation of atherosclerosis caused by 5/6 nephrectomy in apoE-deficient mice. We found that repeated intraperitoneal administration of rhsFlt-1 significantly reduced plaque area and infiltration of plaques by macrophages in the aortas of 5/6-nephrectomized apoE-deficient mice. These findings are consistent with previous studies showing that local adenoviral PlGF-2 delivery promotes atherogenic neointima formation in hypercholesterolemic rabbits, that atherogenic effects are attenuated in apoE and PlGF double-knockout mice,[13] and that an antibody against Flt-1 reduces atherosclerotic plaque growth and vulnerability.[14] We also found that serum indices of uremia and lipid concentrations were higher in 5/6-nephrectomized apoE-deficient mice than in control mice, which is consistent with previous findings.[18] Administration of sFlt-1 reduced atherosclerotic plaque formation without affecting serum urea or lipid levels, but it did significantly reduce infiltration of macrophages into aortic tissues. We therefore suggest that a reduction in circulating sFlt-1 levels in 5/6-nephrectomized apoE-deficient mice worsens atherosclerosis by enhancing the inflammatory processes related to increases in PlGF–Flt-1 signaling. This notion is supported by our previous findings that PlGF is rapidly expressed in myocardial infarct tissue during the acute phase of myocardial infarction and that elevation of plasma PlGF levels stimulates infiltration of monocytes into the myocardium.[31]

Atherosclerosis is more commonly observed in the elder population than in the younger population. In the present study, we adopted the equation for glomerular filtration rate, which is a function of serum creatinine level and age, to investigate the influence of renal dysfunction on the plasma sFlt-1 level. Although there was no significant correlation between plasma level of sFlt-1 and age, it remains to be determined whether other unknown factors, or patients' backgrounds, that would be related to aging or decreased renal function could influence the reduction of circulating sFlt-1 in patients with renal dysfunction.

According to all findings in the present study, we propose the possibility that sFlt-1 plays at least a partial role in the cause-and-effect relationship between renal dysfunction and the worsening of atherosclerosis. Of course, unknown factors related to renal dysfunction or aging, such as enhanced activity of the renin-angiotensin-aldosterone system, en-

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hanced superoxide production,[32] and accumulation of an endogenous inhibitor of nitric oxide synthesis,[33] could be involved in the mechanism for worsening of atherosclerosis in renal dysfunction. Further studies are necessary to better understand the complex mechanism for this.

sFlt-1 has also been studied extensively in the field of obstetrics. In patients with preeclampsia, serum sFlt-1 is elevated to abnormally high levels,[34,35] and Maynard et al[36] showed that such preeclamptic symptoms as hypertension and renal dysfunction could be induced by administration of an adenoviral vector harboring sFlt-1. In the present study, however, administration of rhsFlt-1 did not affect either blood pressure or renal function. This most likely reflects the lower concentrations of sFlt-1 seen after treatment in the present study. In the present study, serum sFlt-1 levels were ≈2 orders of magnitude lower than in the study by Maynard et al.[36]

sFlt-1 also antagonizes VEGF, in part by interrupting signal transduction via Flt-1 and Flk-1.[12,37] It is thus possible that VEGF is involved in the exacerbation of atherosclerosis in patients with renal dysfunction and in 5/6-nephrectomized apoE-deficient mice. In the present clinical study, however, serum VEGF levels did not differ in accordance with renal dysfunction (online-only Data Supplement Figure IIB), and the VEGF/sFlt-1 ratio did not vary as a function of the severity of coronary atherosclerosis (online-only Data Supplement Figure IIF). In the context of the present study, it is therefore unlikely that coronary atherosclerosis was brought about through a relative increase in VEGF.

In conclusion, the present study indicates that a reduction in circulating levels of sFlt-1 in renal dysfunction is associated with the worsening of atherosclerosis.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Chronic kidney disease is a worldwide public health problem not only because it leads to end-stage renal failure but also because it is an independent risk factor for atherosclerosis-related cardiovascular events. Accumulating evidence indicates atherosclerosis is usually worsened in patients with renal dysfunction, and the risk of cardiovascular disease increases sharply as the estimated glomerular filtration rate declines. Additionally, more than 50% of deaths among patients with end-stage renal failure are due to cardiovascular events. Although it is clear that most cardiovascular events in renal dysfunction result from atherosclerosis, the underlying molecular mechanism responsible for the worsening of atherosclerosis in renal dysfunction is not yet fully understood. Consequently, an effective therapeutic strategy is still lacking. Here, we examine the role played by soluble fms-like tyrosine kinase-1 (sFlt-1), an endogenous antagonist of the proatherogenic cytokine placental growth factor (PlGF), in the worsening of atherosclerosis seen in patients with renal dysfunction and in an animal model of renal failure. This report describes our novel observation that circulating sFlt-1 levels are reduced in patients with renal dysfunction in proportion to the severity of the disease, whereas there is no change in plasma PlGF levels. Moreover, renal production of sFlt-1 is also diminished in patients with renal dysfunction, and replacement treatment with recombinant human sFlt-1 reduces five-sixths-nephrectomy–induced worsening of atherosclerosis of apolipoprotein E–deficient mice. Thus, the present findings provide a new insight into the molecular mechanism for worsening of atherosclerosis in renal dysfunction and could lead to a new effective therapeutic strategy.

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SUPPLEMENTAL MATERIAL

Reduction of circulating soluble fms-like tyrosine kinase-1 plays a significant role in renal dysfunction-associated aggravation of atherosclerosis

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Supplemental Methods

Clinical study

Patient classification. We divided the patients into five groups based on renal function: patients whose eGFR was >90 ml/min/1.73 m^2 were categorized in Group 1, those with eGFRs from 60-89 ml/min/1.73 m^2 were in Group 2; those with eGFRs from 30-59 ml/min/1.73 m^2 were in Group 3; those with eGFRs from 15-29 ml/min/1.73 m^2 were in Group 4; and those with eGFRs <15 ml/min/1.73 m^2 were in Group 5.

Gensini's scoring method. Gensini's scoring method is a classification that represents the severity of coronary artery disease (1). The method assigns a score depending on the degree of luminal narrowing and geographical importance of its location; that is, more severe the stenosis and more proximal the location of the lesion in coronary artery, higher the score . Higher scores indicate increased clinical significance.

Blood sampling. We collected blood samples prior to the injection of heparin because we had previously confirmed that circulating levels of sFlt-1 increase by nearly 10-fold immediately after heparin administration.

The Efficacy of ELISA assay and PCR system. As for the enzyme-linked immunoadsorbent assay (ELISA) for sFlt-1, PlGF and VEGF, we used commercially available kits which have been widely used in the world. To minimize inter-assay gap, we always

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measured the same samples for inter-assay control every experiment and re-assayed if the inter-assay control value exceed more than 20 percent of the first control value. The sensitivity and specificity of the ELISA for each assay were described in the manufacturer's instruction. The assay's sensitivity is 3.5 pg/ml, 7.0 pg/ml and 9.0 pg/ml for sFlt-1, PlGF and VEGF respectively. If the measured values were below the limit of detection, they were excluded from statistical analysis. Actually, 15 samples of PlGF and 4 samples of VEGF were excluded, but none of the sFlt-1 samples were excluded from statistical analysis. The assay's specificity is described in the manufacturer's instruction that sFlt-1 assay recognizes only recombinant and natural human sFlt-1 and no significant cross-reactivity is observed with PlGF, VEGF, Flk-1 and so on. As for PlGF, the assay recognizes only recombinant and natural human PlGF and does not have cross-reactivity with VEGF or sFlt-1. As for VEGF, the assay recognizes recombinant and natural human VEGF and does not have cross-reactivity with PlGF or sFlt-1. Although the assay's specificity is described as above, we re-confirmed that the assay for sFlt-1 did not detect recombinant human PlGF and that the assay for PlGF did not detect recombinant human sFlt-1 by ourselves.

We used a specific primer that detected sFlt-1 mRNA. Before the experiment, we confirmed the primer's specificity by using mRNA extracted from human umbilical endothelial cell (HUVEC) as a positive control. As shown in Supplementary Figure 3A, a single band of

PCR product was observed in a dose dependent manner and a single band was also detected in patient's sample. The PCR for human sFlt-1 did not cross-react with full length human Flt-1 as shown in Supplementary Figure 3B. These data indicate that our PCR system works as specific PCR for sFlt-1. The specificity of the primer for mouse sFlt-1 was confirmed in a similar way.

Experimental study

Preparation of recombinant human soluble Flt-1 (rhsFlt-1). A DNA fragment encoding amino acids 1-338 of human Flt1 (sFlt-1 (D1-3)), which contains three Ig-like domains in its N-terminal region, was amplified by PCR using the specific primers 5'-CATCCATGGATCCTGAACTGAGTTTAAAAG-3' (forward) and 5'-CATGGATCCTCAATGTTTCACAGTGATGAATGC-3' (reverse), and human placental cDNA (Clontech Laboratories, Mountain View, CA, USA) serving as the template. The PCR product was subcloned into the pCRII-TOPO vector (Invitrogen), and the sequence was verified by sequencing. The appropriate plasmid was digested with NcoI and BamHI, and the resulting fragment was subcloned into the NcoI/BamHI sites of the pET-30a bacterial expression vector (Novagen, EMD Chemicals). This vector was then used to transform BL21 star (CE3) competent cells (Invitrogen). The transformants were cultured in LB medium containing 50 μg/ml kanamycin in a shaking flask at 37°C until the optical density at 600 nm reached 0.6,

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after which isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, and the culture was continued for an additional 4 h. The cells were then collected by centrifugation and washed, and the pellet was stored at -80°C until further purification of the protein. At that time, the cells were suspended in lysis buffer (50 mM Tris (pH 8.0) containing 100 mM NaCl and 5 mM EDTA) and lysed by incubation with 2 mg/ml lysozyme and sonication. The sFlt-1 (D1-3) protein was present predominantly in inclusion bodies, which were collected by centrifuging the lysate for 20 min at 10,000 rpm. The purified inclusion bodies were solubilized in 20 mM PBS containing 0.5 M NaCl, 6 M urea, 1 mM DTT, and 20 mM imidazole, after which the solution was run over a His-Trap HP (GE Healthcare) column. The eluate was dialyzed against graded concentrations of urea in 20 mM Tris (pH 8.0) buffer containing 0.5 M NaCl. Following the final dialysis against 20 mM Tris (pH 8.0) containing 0.5 M NaCl without urea, the sample was harvested.

The Efficacy of rhsFlt-1 in Vitro and in Vivo. Before the experimental study we confirmed that rhsFlt-1 binds to PlGF *in vitro* and *in vivo*. rhsFlt-1, which contains a histidine-tag, was initially diluted in buffer containing 20 mM imidazole, 0.5 M NaCl, 20 mM Tris, and 0.1 mg/ml BSA to five concentrations: 1 ng/20 μl, 10 ng/20 μl, 100 ng/20 μl, 1000 ng/20 μl, and 10,000 ng/20 μl. The rhsFlt-1 solutions were then mixed with Ni-agarose gel (Qiagen), which binds histidine-tagged sFlt-1. After centrifugation, the supernatant was

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