Table 1. Baseline Characteristics

	Group A	Control A	Group B	Control B	Group C
Number	22	27	12	16	77
Male/female	10/12	15/12	5/7	5/11	42/35
Age (year)	26.8 ± 22.0	28.5 ± 16.2	39.7 ± 23.8	40.0 ± 17.9	50.4 ± 17.2
Gene					
MYH7	4	–	1	–	11
MYBPC3	8	–	1	–	23
TNNT2	3	–	3	–	15
TNNI3	7	–	7	–	28
Echocardiogram					
MaxWT (mm)	9.1 ± 1.8	9.2 ± 1.4	10.3 ± 1.9	9.5 ± 1.2	18.9 ± 4.5*
IVST (mm)	8.7 ± 1.9	9.0 ± 1.7	9.8 ± 2.1	9.1 ± 1.2	17.9 ± 4.7*
PWT (mm)	8.7 ± 1.9	8.7 ± 1.4	9.7 ± 1.8	8.9 ± 1.3	11.3 ± 2.0
LVDd (mm)	42.3 ± 8.4	46.1 ± 4.0	43.9 ± 3.7	45.7 ± 3.5	44.6 ± 5.9
LVDs (mm)	27.6 ± 8.8	28.7 ± 3.8	26.4 ± 6.3	27.6 ± 3.5	28.1 ± 7.2
FS (%)	35.5 ± 7.3	37.7 ± 6.1	39.7 ± 10.8	39.6 ± 6.3	37.7 ± 9.4
LAD (mm)	31.6 ± 9.0	32.2 ± 5.0	35.4 ± 7.2	31.6 ± 5.1	38.3 ± 7.4

*P < 0.001 for comparison of group A, group B, control A and control B.
MaxWT = maximum wall thickness; IVST = interventricular septal wall thickness; PWT = left ventricular posterior wall thickness; LVDd = left ventricular end-diastolic dimensions; LVDs = left ventricular end-systolic dimensions; FS = left ventricular fractional shortening; LAD = left atrial dimensions.

dispersion were significantly longer in group C in comparison with those in group A (Figs. 2). Interestingly, QT dispersion in group B was also significantly increased over that in group A and that in age-matched controls (control B). In contrast, QT variables in group A were not different from those in age-matched controls (control A). As for T-peak to T-end interval, there was no significant difference among these groups (Table 2).

To make sure of the relationship between the QT variables and clinical outcome, we have followed mutation carriers prospectively. Fifty-seven of 111 mutation carriers including 13 of group A, 6 of group B, and 38 of group C could be followed. Mean follow-up periods were 6.9 years (1–15 years). There were four cases of SCDs and one case that was resuscitated from ventricular fibrillation in group C. ICD was implanted to the resuscitated patient after this episode. However, there was no cardiac death in group A and group B, although two nonlethal arrhythmias such as triplet PVC were detected by 24-hour Holter monitoring in group B.

Interestingly, T-peak to T-end interval of the five subjects who had SCD or ventricular fibrillation were 188 ± 22 ms, which tended to be longer than those from the remaining subjects without SCD or ventricular fibrillation in the group C (165 ± 46 ms). It is important that LV function of five patients suffering from SCD or ventricular fibrillation

Table 2. QT Variables in Each Group

	Group A	Control A	Group B	Control B	Group C
RR (ms)	880 ± 189	899 ± 153	935 ± 152	922 ± 127	959 ± 146
MaxQT (ms)	379 ± 41	387 ± 36	403 ± 40	386 ± 26	427 ± 47**
MinQT (ms)	338 ± 37	341 ± 27	342 ± 33	340 ± 23	366 ± 43**
QT dispersion (ms)	42 ± 19	46 ± 16	61 ± 22*	45 ± 17	62 ± 31**
MaxQTc ($ms^{1/2}$)	408 ± 29	410 ± 31	419 ± 37*	403 ± 20	437 ± 35**
MinQTc ($ms^{1/2}$)	363 ± 31	362 ± 24	355 ± 25	356 ± 22	375 ± 36
QTc dispersion ($ms^{1/2}$)	45 ± 18	49 ± 17	64 ± 23*	47 ± 16	62 ± 27**
T-peak to T-end (ms)	173 ± 35	152 ± 48	175 ± 24	163 ± 29	168 ± 47

*P < 0.05 for comparison of group A; **P < 0.01 for comparison of group A.
RR = RR interval; MaxQT = maximum QT interval; MinQT = minimum QT interval; MaxQTc = corrected maximum QT interval; MinQTc = corrected minimum QT interval; QTc dispersion = corrected QT dispersion; T-peak to T-end = T-peak to T-end interval.

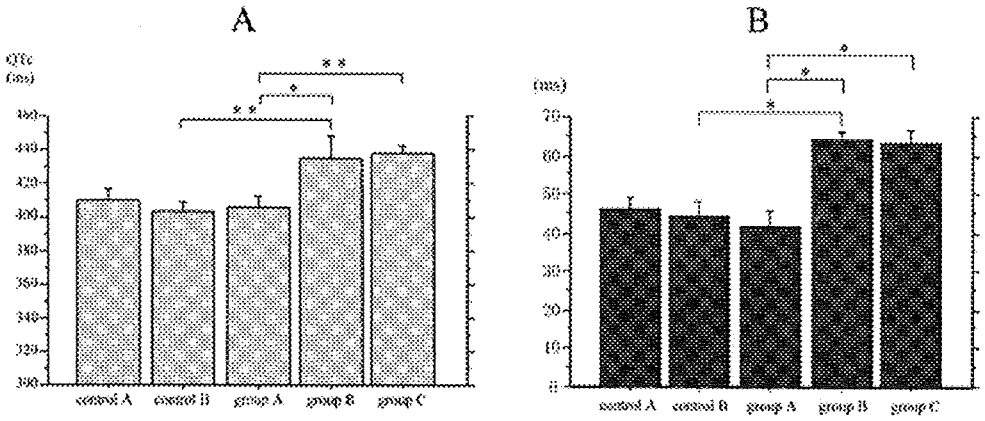

Figure 2. Maximum QTc interval (maxQTc) and QT dispersion in each group. (A) Max QTc of group B and group C was significantly longer than group A. (B) QT dispersion of group B and group C also increased than group A. *P < 0.05 for comparison to group A; **P < 0.01 for comparison to group A.

was not impaired in comparison with the remaining patients.

DISCUSSION

The present study demonstrates that, in genotyped subjects, QT variables can be altered not only in subjects with LVH but also in those without LVH accompanying any ECG abnormalities such as abnormal Q wave, high voltage and inverted T wave. Under these conditions, lethal or nonlethal ventricular arrhythmia can occur in those subjects even without LVH.

In the present study QT variables did not change in mutation carriers lacking ECG abnormalities (group A), but were changed in those with ECG abnormalities even in absence of LVH (group B), although previous studies have revealed that HCM patients have longer maximum QTc interval and increased QT dispersion with respect to values in normal controls.[16,23] Jouven et al.[24] analyzed QT variables in mutation carriers in the beta-myosin heavy-chain or myosin-binding protein-C gene, and reported that QT dispersion in mutation carriers without either ECG abnormalities or wall hypertrophy (nonpenetrants) did not differ from that in controls, as observed in the present study. However, maximum QTc interval in non-penetrants was increased over that in controls, although QTc interval was not prolonged in our group A subjects. This discrepancy may be due to differences in genes involved or in criteria for ECG abnormalities. Actually, in our group A subjects, the mutation of the beta-myosin heavy-chain gene or the myosin-binding protein-C gene was observed in only 12 out of 22. QT dispersion may be changed in association with appearance of any ECG abnormalities in mutation carriers even in the absence of prominent LVH.

QT dispersion is thought to reflect regional heterogeneity of ventricular repolarization, and it is thought that upper limit of 50 ms is highly specific.[25] Prolonged QT dispersion correlates with the incidence of ventricular tachyarrhythmias and sudden death in patients with HCM,[4,5] and usually precedes the appearance of LVH.[21] Before myocyte hypertrophy develops, electrophysiological activity may be changed in cardiomyopathic cells and may produce heterogeneities of ventricular depolarization and repolarization.

The relationship between QTc interval and left ventricular wall thickness was reported to be different between mutation genes,[24] and LVH in athletes was not associated with prolonged QTc interval.[26] This suggests that gene mutation itself may affect QTc interval independent of myocardial hypertrophy. Additionally, inhomogeneities of LV wall thickness also influence QT dispersion.[27] Therefore, in the advanced stages, morphological inhomogeneities such as asymmetrical septal hypertrophy as observed in the present study, in addition to electrical inhomogeneity at the myocyte level, may contribute to increasing QT dispersion.

Shimizu et al. reported that T-peak to T-end interval favorably reflect the transmural dispersion of repolarization because the end of repolarization

of the epicardial cell corresponded to the peak of the T wave, whereas the end of repolarization of the M cell corresponded to the end of the T wave.[17] Indeed, we previously reported that prolonged T-peak to T-end interval was associated with SCD or malignant VT in the genotyped HCM subjects with troponin I gene mutation.[6] However, in the present study there was no difference in T-peak to T-end interval between studied groups. This may be explained by the fact that the number of the cases with SCD or malignant VTs was small and different kinds of genotyped patients were included for analysis. Even under these conditions, it is interesting that the five subjects with SCD or ventricular fibrillation during follow-up period exhibited prolonged T-peak to T-end without statistical significance.

Clinical Implications and Limitation

The present results suggest that we must be aware of the potential for occurrence of lethal arrhythmias/sudden cardiac death in individuals carrying the disease-causing gene mutation of HCM when any ECG abnormalities are observed. To test this hypothesis, the enrolled patients were followed up over an average of 6.9 years. Surprisingly, we identified five patients from group C (1.64%/year) and two patients from group B suffered from sudden cardiac death or ventricular arrhythmias during follow-up periods. Previous study pointed out that the annual rate of sudden death or ICD discharge was 1.02% in clinical HCM patients with evident LVH.[28] The present data suggest that mutation carriers may suffer from serious cardiac events if they have increased maximal QTc interval and/or QT dispersion even without LVH.

In the present study, we specifically examined subjects with HCM-related gene mutation carriers. However, we did not consider the effects of conventional medical treatment, although the use of amiodarone and/or ICD was avoided until the occurrence of cardiac event. Further study with medical intervention may demonstrate clinical significance of QT variables in the occurrence of lethal cardiac events in genotyped subjects with ECG abnormalities.

CONCLUSION

Maximal QTc interval and/or QT dispersion increased in HCM-related gene mutation carriers with ECG abnormalities in the absence or presence of LVH. We suggest the possible occurrence of lethal arrhythmia in mutation carriers of HCM when ECG abnormalities including QT variables are observed before the appearance of LVH.

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Nifedipine inhibits the activation of inflammatory and immune reactions in viral myocarditis

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ABSTRACT

Aims: The aim of study is to investigate the effect of nifedipine on viral myocarditis in an animal model.
Main methods: Four-week-old male DBA/2 mice were inoculated with 2 pfu of encephalomyocarditis virus (EMCV) and randomized to nifedipine ($n=10$) or control ($n=10$) group. The control group was fed by regular chow and the nifedipine group contained 0.01% of nifedipine. Mast cell density was counted, and expressions of messenger RNAs of stem cell factor (SCF), matrix metalloproteinases (MMPs), pro-collagen I, mast cell proteases, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6) were evaluated by RT-PCR.
Key findings: The area of myocardial necrosis was smaller in the nifedipine vs the control group (mean ± SD, 1.2 ± 1.3% vs 3.8 ± 1.8%, respectively, $P<0.005$). The mast cell density (count/mm^2) was lower in the nifedipine vs the control group (mean ± SD, 0.23 ± 0.16 vs 1.08 ± 0.45, respectively, $P<0.0005$). The expressions of MMPs, mast cell proteases, TNF-α, IL-6, SCF and pro-collagen I were lower in the nifedipine group than in the control group ($P<0.05$).
Significance: Nifedipine inhibited the activation of various participants in inflammatory and immune reactions in EMCV myocarditis.

Introduction

Viral infection is the most common cause of myocarditis. Viral myocarditis can occur as an acute fulminant illness or a chronic disorder that leads to dilated cardiomyopathy. Acute viral myocarditis is an important cause of cardiac failure in youth. But, there is no definite effective treatment apart from general heart failure therapy. Encephalomyocarditis virus (EMCV) is a picornavirus that can cause myocarditis within days of infection. We have studied the histological changes and expressions of matrix metalloproteinases (MMPs), collagen, mast cell proteases and proinflammatory cytokines in EMCV myocarditis in previous studies (Higuchi et al. 2008; Kitaura-Inenaga et al. 2003; Matsumori et al. 1991).

The calcium channel blocker nifedipine is effective in heart failure (Okuda et al. 2005), and beneficial for myocardial metabolism (Berkels et al. 2001). Several *in vitro* studies have shown that calcium channel blockers suppress the activation of various participants in immune reactions, including T cells (Bacon et al. 1989; Birx et al. 1984), mast cells (Tanizaki et al. 1983) and macrophages (Shen et al. 1995; Wright et al. 1985), via the inhibition of calcium influx, suggesting that they can be immunosuppressant. The aim of study was to investigate the effect of nifedipine on viral myocarditis in an animal model.

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Materials and methods

Study design

Four-week-old male DBA/2 mice were inoculated with 2 plaque forming units of EMCV and randomized to the nifedipine ($n=10$) or control ($n=10$) groups. The control group was fed regular chow and the nifedipine group was fed chow containing 0.01% nifedipine, begun on the day of inoculation. The mice were sacrificed on day 5. This investigation confirms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). The percentage of necrosis area/total area of the apical myocardium was observed using Masson's trichrome staining. Mast cell density (count/mm^2) was counted, and expressions of mRNA of stem cell factor (SCF), matrix metalloproteinases, collagen, mast cell proteases 4, 5 and 6 and proinflammatory cytokines TNF-α and IL-6, inducible nitric oxide syntase (iNOS), and Toll-like receptor 4 (TLR4) were evaluated by real-time quantitative RT-PCR.

Histological analysis

The hearts were fixed with neutral buffered formalin, embedded in paraffin, sectioned at a thickness of 5 μm, and stained with hematoxylin–eosin for overall morphology and with Masson's trichrome stain. The size of the myocardial lesion was determined with Microanalysis (Ather Corporation, Tokyo, Japan), and the percentage of necrotic area per total

cardiac area was determined by microscopic slides stained with Masson's trichrome stain. To determine the number of mast cells, the hearts were stained with toluidine blue. The total number of mast cells in a given section (whole heart) was calculated as cells/mm^2.

Quantitative reverse transcription-PCR

Total RNA was extracted from the myocardial samples with TRIzol according to the recommendations of the manufacturer. Briefly, myocardial samples were lysed in TRIzol, and chloroform was added, followed by phased separation by centrifugation. RNA was precipitated with isopropanol and pelleted by centrifugation. Pellets were washed with 80% ethanol and redissolved in RNA-free water. For cDNA generation, 5 μg of RNA was subjected to reverse transcription with oligo(dT) as a primer and expand reverse transcriptase. The cDNA was amplified by PCR using the primers shown in Table 1. The conditions for the TaqMan PCR were: 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min.

Statistical analysis

All results are expressed as mean ± SD. Differences between the 2 groups were tested by unpaired two-tailed Student's test. Differences were considered statistically significant at $P<0.05$.

Results

Effect of nifedipine on area of myocardial necrosis

Nifedipine ingestion for 5 days after EMCV inoculation reduced the area of myocardial necrosis compared to that for control (mean ± SD, 1.2 ± 1.3% vs 3.8 ± 1.8%, respectively, $P<0.005$; Fig. 1).

Table 1
RT-PCR primers.

Genes	Primers
mMCP-4 F	5′-GAAGTGAAAAGCCTGACCTGC-3′
mMCP-4 R	5′-CATGCTTTGTTGAACCCAAGG-3′
mMCP-4 P	5′-TGCATCAGAGTCTTCAAGCCAGAGCTC-3′
mMCP-5 F	5′-TTGCCAGCCTGTCAGCAAA-3′
mMCP-5 R	5′-TACAGACAGGCCAGATCGCAT-3′
mMCP-5 P	5′-CTGGAACTGGAATAGTGCAGGTTTTGTGTG-3′
mMCP-6 F	5′-CGACATTGATAATGACGAGCCTC-3′
mMCP-6 R	5′-ACAGGCTGTTTTCCACAATGG-3′
mMCP-6 P	5′-CCCACCTCCTTATCCTCTGAAGCAAGTGA-3′
Pro-a-2(I) collagen F	5′-GAGGACACCCCTTCTACGTTGTA-3′
Pro-a-2(I) collagen R	5′-CAGTCCAACAAGCATGTCTGGT-3′
Pro-a-2(I) collagen P	5′-CAAACTGGCTGCCACCATTGATAGTCTCTC-3′
MMP-9 F	5′-TTGTGGTCTTCCCCAAAGACC-3′
MMP-9 R	5′-TATCCACCGAGCCATCTGTCTA-3′
MMP-9 P	5′-AAAACCTCCAACCTCACGGACACCCA-3′
MT-MMP-2 F	5′-ACTGACCTGCATGGAATCAGC-3′
MT-MMP-2 R	5′-GGTTACTTGAGTGTTCTAGCCCA-3′
MT-MMP-2 P	5′-TCTTTCTGGTGGCCGTGCATGA-3′
GAPDH F	5′-TTCACCACCATGGAGAAGGC-3′
GAPDH R	5′-GGCATGGACTGTGGTCATGA-3′
GAPDH P	5′-TGCATCCTGCACCACCAACTGCTTAG-3′
TNF-αF	5′-CATCTTCTCAAAATTCGAGTGACAA-3′
TNF-αR	5′-TGGGAGTAGACAAGGTACAACCC-3′
TNF-αP	5′-CACGTCGTAGCAAACCACCAAGTGGA-3′
IL-6F	Based on TaqMan product no. 4331348
IL-6R	Based on TaqMan product no. 4331348
IL-6P	Based on TaqMan product no. 4331348
iNOS F	5′-CAGCTGGGCTGTACAAACCTT-3′
iNOS R	5′-CATTGGAAGTGAAGCGTTTCG-3′
iNOS P	5′-CGGGCAGCCTGTGAGACCTTTGA-3′
TLR4 F	Based on TaqMan product no. 445274
TLR4 R	Based on TaqMan product no. 445274
TLR4 P	Based on TaqMan product no. 445274

F: forward, R: reverse, P: probes.

Effect of nifedipine on mask cell density and its proteases

Mast cell density (count/mm^2) was lower in the nifedipine group than in the control group (mean ± SD, 0.23 ± 0.16 vs 1.1 ± 0.45, respectively, $P<0.0005$) at 5 days after nifedipine ingestion (Fig. 2). The expression of MCP-4, MCP-5 and MCP-6 was lower in the nifedipine than in the control group [$(0.12\pm0.01)\times10^{-2}$ vs $(1.1\pm1.1)\times10^{-2}$, $(0.65\pm0.56)\times10^{-2}$ vs $(5.4\pm5.2)\times10^{-2}$, and $(1.1\pm1.0)\times10^{-3}$ vs $(2.6\pm1.9)\times10^{-3}$, respectively, $P<0.05$; Fig. 3].

Effect of nifedipine on pro-collagen I and MMPs

Expressions of pro-collagen I, MMP-9, and MMP-2 was lower in the nifedipine group than in the control group [$(1.6\pm1.0$ vs $4.5\pm3.2)\times10^{-2}$, $(0.37\pm0.3)\times10^{-2}$ vs $(3.9\pm4.8)\times10^{-2}$, and $(0.83\pm0.93)\times10^{-5}$ vs $(1.9\pm1.3)\times10^{-5}$, respectively, $P<0.05$; Fig. 4].

Effect of nifedipine on proinflammatory cytokines and SCF

Expressions of the proinflammatory cytokines TNF-α, IL-6 and SCF were also lower in the nifedipine than in the control group [$(0.21\pm0.14)\times10^{-7}$ vs $(2.9\pm2.4)\times10^{-7}$, $(0.99\pm0.74)\times10^{-4}$ vs $(14\pm13)\times10^{-4}$, and $(1.8\pm1.3)\times10^{-7}$ vs $(8.4\pm8.2)\times10^{-7}$, respectively, $P<0.05$; Fig. 5].

Effect of nifedipine on iNOS and TLR4

Expressions of iNOS and TLR4 tended to be lower in the nifedipine than in the control group, but the differences did not reach statistical significance;
iNOS, $(0.069\pm0.063)\times10^{-4}$ vs $(1.3\pm2.1)\times10^{-4}$, $P=0.078$,
TLR4, $(0.86\pm0.76)\times10^{-6}$ vs $(2.4\pm3.1)\times10^{-6}$, $P=0.14$.

Discussion

Effect of nifedipine on MMPs, mast cell and mast cell proteases

Matrix metalloproteinases, in the family of zinc endopeptidases, are the major regulators of collagen degradation and all constituents of the matrix involved in the pathogenesis of several diseases of the heart, and are implicated in cardiac repair and remodeling after cardiac injury or stress. The expressions of MMPs and collagen were increased in virus myocarditis in a previous study (Kitaura-Inenaga et al. 2003).

MMP-9 has been reported to be secreted by most immune cells to aid matrix degradation during migration. Immune cells secrete proinflammatory cytokines, such as TNF-α and interleukin-1, which may trigger further MMP up-regulation. MMP-9 has been suggested to play a role in cytokine processing through cleavage and release of the active molecules, in particular, TGF-β and interleukins. The fact that this protease is increased only during the inflammatory phase supports the suggestion that it modulates inflammation and early healing, possibly through cytokine regulation (Cheung et al. 2006).

MMP-2 has overlapping yet distinct functions relative to MMP-9, as evidenced by the difference in localization of these proteases in the heart. Whereas MMP-9 is localized to infiltrating immune cells, MMP-2 is more widely distributed and is found in necrotic foci and remote cardiomyocytes (Kwan et al. 2004). Increased levels of MMP-2 may influence cytokine regulation, cell-to-cell/matrix contacts, and cell migration, all of which are necessary during wound healing. Recently, MMP-2 was shown to have intracellular functions, where MMP-2 was localized to the sarcomeres and nucleus, possibly resulting in myocyte dysfunction (Kwan et al. 2004). Regulation of MMP-2 and MMP-9, in myocarditic hearts is a result of both differential gene expression and inhibition by TIMPs. These proteases not only function in degradation

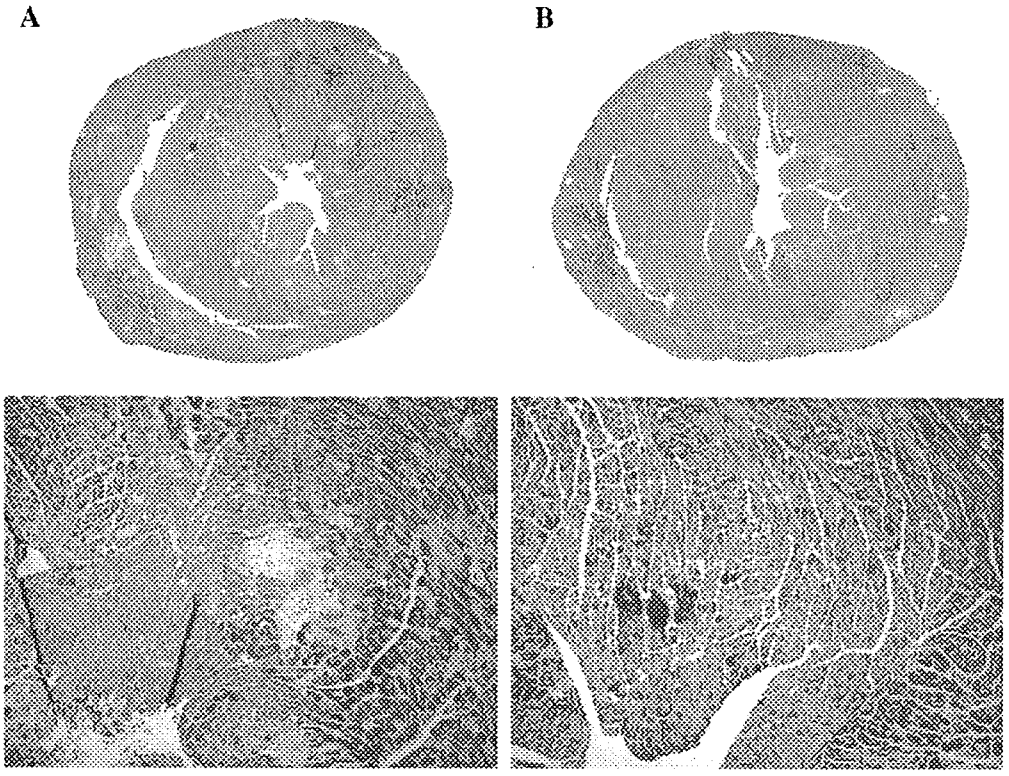

Fig. 1. Myocardial necrosis in nifedipine and control. A: Control group, and B: nifedipine group. The upper panel is 20×, and the lower panel 100×. The grey areas are necrotic tissue, and show that there is less necrosis in Panel B.

of matrix but also act as major regulators of the cytokine profile, angiogenesis, and wound healing, contributing to myocardial injury and remodeling during the progression of myocarditis (Cheung et al. 2006). The decrease in expression of MMP-2, MMP-9, and pro-collagen I after 5 days of nifedipine after EMCV inoculation suggests that nifedipine mitigated myocardial injury and remodeling during the progression of myocarditis.

Mast cells are multifunctional cells that contain various mediators such as cytokines, histamine, proteases and leukotrienes. They are found in nearly all major organs of the body and are involved in many types of inflammation, including allergic inflammation. Mast cells are also found in the human heart and have been implicated in cardiovascular diseases (Hara et al. 2002; Matsumori 2004). Mast cell chymase induces the proliferation of cardiac fibroblasts and apoptosis of cardiac myocytes (Hara et al. 1999). We have previously reported that IL-1β, a prominent cytokine in cardiac remodeling, is up-regulated in the chronic stage of EMCV myocarditis (Shioi et al. 1996), and it has also been implicated in fibrosis after myocardial infarction (Ono et al. 1998). Mast cells increase in number in the failing heart (Patella et al. 1998). In a rat model of acute myocardial infarction, the mast cell density reached its peak on day 21, when cardiac remodeling was ongoing (Engels et al. 1995).

We found that the number of mast cells was increased significantly when myocardial fibrosis became apparent, and the gene expressions of mMCP-4, -5, and -6, MMP-9, and type-I pro-collagen were markedly increased in a previous study (Kitaura-Inenaga et al. 2003). There are other similar reports (Meng et al. 2004). These observations suggest that mast cells participate in the progression of heart failure and cardiac remodeling, and that mast cell chymase (MCP-4 and -5) and tryptase (MCP-6) may be implicated in both the acute inflammatory reaction and the remodeling process associated with acute viral myocarditis. More recently, we have shown that mast cells and their mediators play a critical role in the pathogenesis of viral myocarditis (Higuchi et al. 2008). It appears that nifedipine suppressed cardiac injury in myocarditis in the present study, as shown by the expression of mast cell proteases (MCP-4, -5 and -6) and the lower mast cell density.

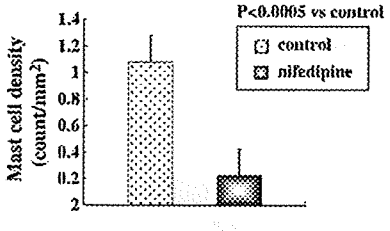

Fig. 2. Mast cell density. The mast cell density is lower in the nifedipine group than in the control group.

Effects of nifedipine on TNF-α, Il-6, and NF-κB

The proinflammatory cytokines TNF-α and Il-6 are known to be increased in congestive heart failure, which can be induced by inflammatory cytokines contributing to the progressive myocardial damage in myocarditis because of stimulation of cardiac fibrosis and induction of apoptosis as a part of the overall remodeling process (Ishiyama et al. 1997).

NF-κB regulates the expression of several genes involved in immune and inflammatory responses for example TNF-α and IL-6 (Kopp and Ghosh 1995). NF-κB is not the only transcription factor involved in regulating these genes, however, and it often operates

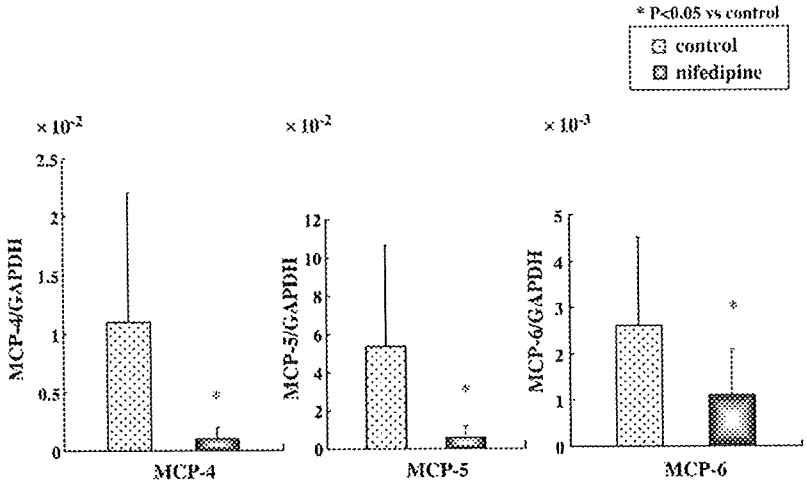

Fig. 3. Expression of MCPs in nifedipine and control groups. In the nifedipine group, the expressions of mast cell proteases were lower than in the control group.

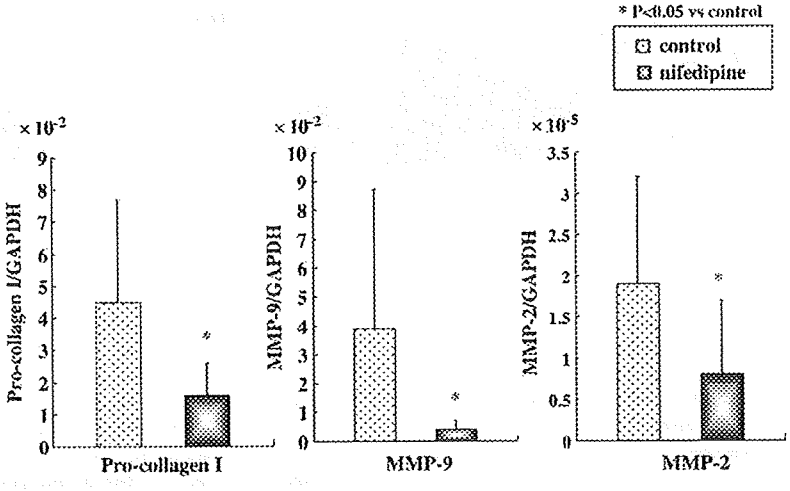

Fig. 4. Expression of pro-collagen I, and MMPs in the nifedipine and control groups. Pro-collagen I and MMPs (MMP-2 and MMP-9) are lower in the nifedipine group compared to the control group.

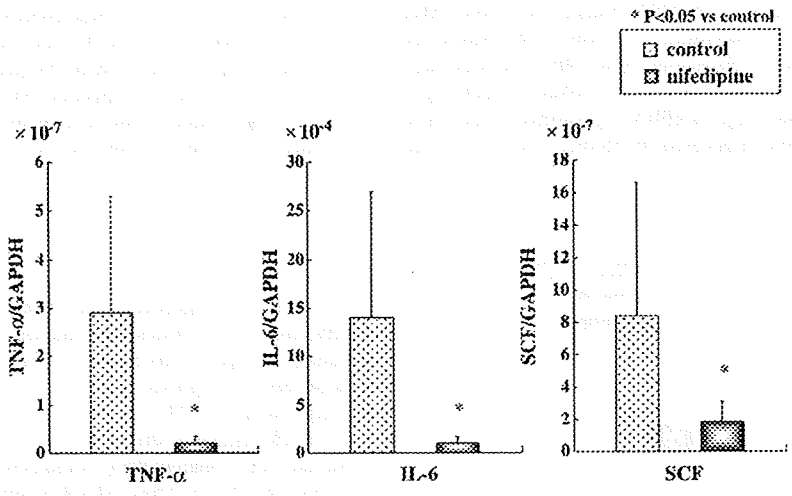

Fig. 5. Expression of TNF-α, IL-6 and SCF in the nifedipine and control groups. TNF-α, IL-6 and SCF were lower in the nifedipine group than in the control group.

with other transcription factors, such as activator protein 1 (AP-1) and the nuclear factor of IL-6, which are also involved in the regulation of inflammatory and immune genes (Barnes and Karin,1997; Stein and Baldwin 1993; Stein et al. 1993). NF-κB is activated by many of the factors which promote the inflammatory response, including viral infections, oxidants, and antigens. This activation, in turn, leads to the coordinated expression of several genes that encode proteins (such as cytokines, chemokines, adhesion molecules, and enzymes) involved in mediator synthesis and further amplification and perpetuation of the inflammatory response. The present data showed that the proinflammatory cytokines TNF-α and IL-6 were decreased after administering nifedipine, implying that it inhibited the activation of NF-κB.

We found that nifedipine inhibited the activation of NF-κB in a human epithelial-like lung carcinoma cell line (Matsumori et al. 2000). Those findings differ from previous reports of an activation of NF-IL-6 and NF-κB by calcium channel blockers such as amlodipine, diltiazem, and verapamil (Eickelberg et al. 1999) although amlodipine showed beneficial effect on myocarditis (Veeraveedu et al. 2006; Wang et al. 1997). The precise explanation for the effect of nifedipine on viral myocarditis remains to be found, though some data have suggested that the role of NF-κB in cytokine signaling and associated cellular responses are cell- and tissue-specific (Baeuerle and Henkel 1994; Gerondakis et al. 1998; May and Ghosh 1998). It has been shown that IκB kinase activity. In response to lipopolysaccharide was markedly inhibited by Ca^{2+} modulators including nifedipine (Kim et al. 2004), and that a cell-permeable Ca^{2+} chelator, BAPTA-AM, reduced cytosolic Ca^{2+} concomitant with a reduction in NF-κB binding activity and a reduction in IκB kinase activity (Petranka et al. 2001). These observations suggest that nifedipine may act in a signal transduction pathway upstream of IκB kinase to mediate IκB phosphorylation (Wu et al. 2006). Since activation of NF-κB has also been evoked in the development of rheumatoid arthritis, autoimmune diseases, atherosclerosis and neoplasms (Baldwin 1996), nifedipine may also have a modulating effect in these disorders.

It has been reported that TLR4 and myeloid differentiation factor-88 play important roles in the pathogenesis of viral myocarditis (Fairweather et al. 2003; Fuse et al. 2005), and myeloid differentiation factor-88 mediated activation of NF-κB by TLR4 (Bhattacharyya et al. 2008). Our study showed that expression of TLR4 tended to decrease in the heart with viral myocarditis, which might contribute to improvement of myocardial injury.

We have previously studied the effects of various drugs which have the effects of left ventricular unloading, and found differential effects by these agents. An α-adrenergic blocking agent, and a β-adrenergic agent did not have a beneficial effect on the same animal model of viral myocarditis (Matsumori 1993; Tominaga et al. 1991). Therefore, left ventricular unloading did not reduce myocardial injury.

The dose of nifedipine used in this study is comparable to 15 mg/kg/day. It is difficult to compare doses used in different animal species, but on the basis of body surface area, a given dose in mice is comparable to a dose that is 12-times lower in humans (Chodera and Feller 1978). Thus, the dose of 15 mg/kg in mice is equivalent to a dose of 1.25 mg/kg in humans, which is within the range of clinical dosing.

Conclusions

Nifedipine inhibited the activation of various participants in inflammatory and immune reactions in EMCV myocarditis, and is a promising agent for the treatment of viral myocarditis.

Acknowledgment

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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 atheroscle-

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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]

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,

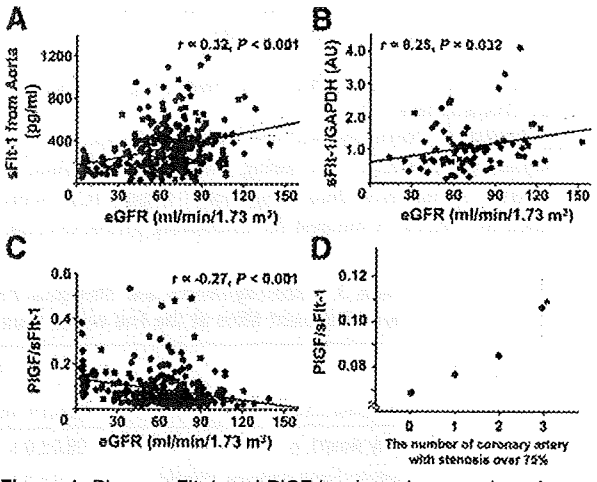

Figure 1. Plasma sFlt-1 and PlGF levels and expression of sFlt-1 mRNA. A, A significantly positive correlation was found between plasma levels of sFlt-1 from aorta and eGFR. n=329; r=0.32; P<0.001. B, A significantly positive correlation was found between relative levels of sFlt-1 mRNA in human renal 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.

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, $P<0.0011$; 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.

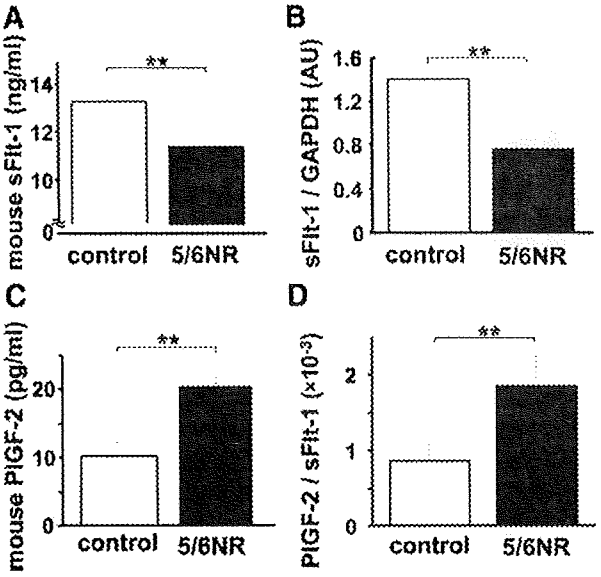

Figure 2. The effect of 5/6 nephrectomy (5/6NR) on serum sFlt-1 and PlGF-2 levels and renal expression of sFlt-1 mRNA in apoE-deficient mice. A and B, Both serum sFlt-1 levels (A) and renal sFlt-1 mRNA expression (B) were significantly lower in 5/6-nephrectomized apoE-deficient mice than in control apoE-deficient 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.

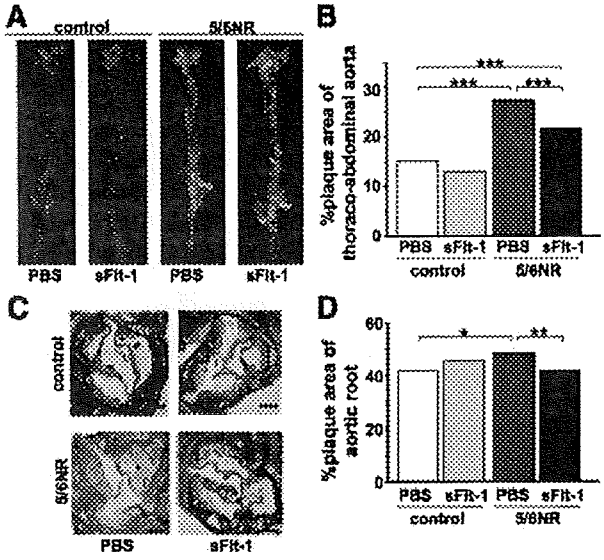

Figure 3. Atherosclerotic plaque formation in thoracoabdominal aorta and aortic root in apoE-deficient mice. Plaque formation in the thoracoabdominal aorta (A and B) and aortic root (C and D) was exacerbated by concomitant renal dysfunction and was mitigated to some extent by repeated intraperitoneal 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 $\times10^{-3}$; $P<0.01$; 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

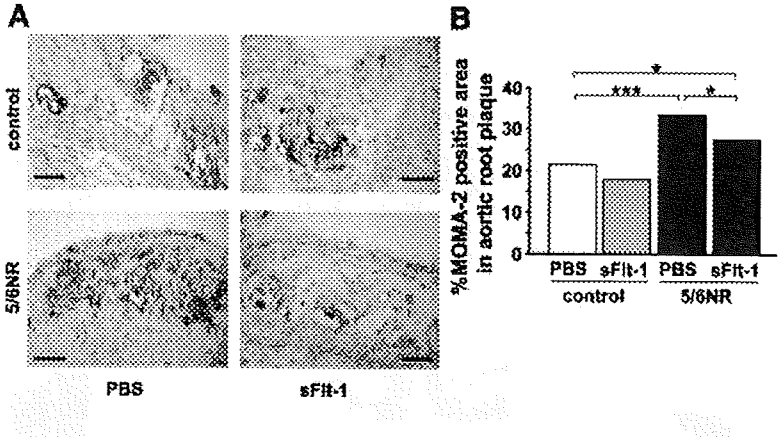

Figure 4. Infiltration of macrophages into atherosclerotic plaque in aortic roots from apoE-deficient mice. Infiltrating macrophages were stained with MOMA-2 (A). Infiltration was enhanced by progression of renal dysfunction and was reduced somewhat by repeated intraperitoneal injection of rhsFlt-1 (A 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-

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.

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