Plasma concentration of coupling factor 6 and cardiovascular events in patients with end-stage renal disease

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Background. Plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), is an independent predictor of overall mortality and cardiovascular outcome in hemodialysis patients. However, not only ADMA but also traditional risk factors account for only part of the high cardiovascular morbidity and mortality in these patients. We investigated cross-sectionally the association between coupling factor 6 (CF6), an endogenous inhibitor of prostacyclin synthesis, and cardiovascular events in 95 hemodialysis patients.

Methods. Plasma CF6 level was measured by radioimmunoassay, whereas plasma ADMA level by high-performance liquid chromatography (HPLC).

Results. Plasma levels of CF6 and ADMA were threefold higher in hemodialysis patients than in control individuals, and there was a positive correlation between these two compounds ($r = 0.25$, $P < 0.05$). Plasma CF6 level was positively correlated with serum creatinine level ($r = 0.36$, $P < 0.01$) and was reduced after dialysis ($P < 0.05$). Plasma CF6 and ADMA levels were both higher in hemodialysis patients complicating ischemic heart disease (myocardial infarction and/or angina) than in those free of cardiovascular events. In a multiple regression model, plasma CF6 level ($r = 0.24$, $P = 0.023$) and ADMA level ($r = 0.26$, $P = 0.023$) were independently related to the occurrence of ischemic heart disease in hemodialysis patients.

Conclusion. CF6 is a novel risk factor for ischemic heart disease in end-stage renal disease (ESRD). Synergism of this peptide and ADMA might contribute to its occurrence presumably by inhibition of prostacyclin and nitric oxide production. A prospective study is needed to evaluate this issue more precisely.

Key words: coupling factor 6, asymmetric dimethylarginine, end-stage renal disease, cardiovascular event.

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Cardiovascular disease is a major cause of death in patients with end-stage renal disease (ESRD). The projected life expectancy of patients on dialysis is 20% to 25% of that of the general population [1]. Although patients with ESRD commonly have associated diseases that have a high cardiovascular risk in themselves, such traditional risk factors account for only parts of the high cardiovascular morbidity and mortality in these patients [1]. A recent prospective study showed that plasma asymmetric dimethylarginine (ADMA), an endogenous inhibitor of nitric oxide synthase (NOS), was an independent predictor of overall mortality and cardiovascular outcome in hemodialysis patients [2]. The plasma ADMA level was reported to be two to six times higher in uremic patients than in healthy control individuals [3–5], and to be higher in dialysis patients with clinically manifested atherosclerosis than in those without atherosclerotic disease [5]. However, this compound could not account fully for the high cardiovascular morbidity and mortality.

Recently, coupling factor 6 (CF6), an essential component of the energy-transducing stalk of mitochondrial adenosine triphosphate (ATP) synthase [6], was found as a novel endogenous inhibitor of prostacyclin synthesis [7]. It was identified while investigating the mechanism of suppression of prostacyclin in spontaneously hypertensive rats (SHR). Levels of circulating prostacyclin in SHR were decreased compared with those in normotensive control, despite the fact that, as measured in isolated aortic strips, prostacyclin generation was elevated in SHR [8, 9]. We showed that CF6 suppresses cytosolic phospholipase A$_2$ in vascular endothelial cells [7] and functions as an endogenous vasoconstrictor in the fashion of a circulating hormone [10]. We hypothesized that CF6 is not excreted in patients with chronic renal failure, and its plasma concentration is increased in uremic patients. Thus, accumulation of CF6 might be a novel cardiovascular risk factor in ESRD. In this study, we investigated the association between CF6 and cardiovascular events in patients with ESRD.
METHODS

Subjects

This study protocol was approved by the Ethics Committee of the University Hospital, and was performed after informed consent was obtained from each subject. Ninety-five patients with ESRD (52 men and 43 women with a mean age of 58 years) who had undergone regular dialysis treatment for at least 6 months and without clinical evidence of circulatory congestion were investigated. The patients were treated with standard dialysis three times a week. The quantity of blood was 170 ± 3 mL/min, and the quantity of dialysate was all 500 mL/min. The duration of dialysis was 3.8 ± 0.1 hours. Dialyzer used in this study was polysulfone (53%), polymethylmethacrylate (41%), and vitamin E-coated polysulfone (6%). Kt/V was 1.13 ± 0.03. Dry weight was targeted in every patient to achieve a normotensive edema-free state. Blood was drawn at a midweek interval for the measurements of plasma levels of CF6, ADMA, and biochemical items after 20 to 30 minutes of quiet resting in a semirecumbent position prior to dialysis treatment. In ten patients, blood was also drawn for the measurement of CF6 level after dialysis treatment. In only eight patients, urine was excreted once a few days (the average of daily volume <200 mL) and was collected for the measurement of CF6 level. Cardiovascular risk factors and cardiovascular events were assessed in each patient. The diagnosis of arrhythmia, most of which was atrial fibrillation, was made by the ECG. The diagnosis of acute myocardial infarction (AMI) was made by persistent chest pain typical of AMI, ST segment elevation >1.0 mm on ≥2 leads after exercise testing or during spontaneous chest pain attack in the ECG. The diagnosis of acute myocardial infarction (AMI) was made by persistent chest pain typical of AMI, ST segment elevation >1.0 mm on ≥2 leads, and elevation of cardiac enzymes. The diagnosis of arrhythmia, most of which was atrial fibrillation, was made by the ECG. The diagnosis of peripheral artery disease was made by its typical symptom and digital pulse volume recording. The diagnosis of retinal vein thrombosis was made by a dilated fundus examination.

In another group of 13 patients with chronic renal failure who did not undergo maintenance dialysis (ten men and three women with a mean age of 58 years), blood was drawn for the measurement of CF6. In 27 control subjects with normal renal function (15 men and 12 women with a mean age of 51 years), blood and urine were collected for the measurement of CF6.

Radioimmunoassay for CF6

Sep-Pak C18 cartridges loaded with plasma or urine acidified with HCl. After washing with water, the absorbed materials were eluted with 60% acetonitrile containing 0.1% trifluoroacetic acid (TFA) and submitted to radioimmunoassay (RIA). The standard recombinant CF6, which was obtained from Escherichia coli using a cleavable fusion protein strategy [11], or the unknown samples was incubated with anti-CF6 antiserum diluent (1:1200) for 12 hours, and then the tracer solution (18,000 to 20,000 cpm) was added. After incubation for 24 hours, antirabbit IgG goat serum diluent containing 10% polyethylene glycol 6000 and rabbit IgG were added and radioactivity of the precipitate was measured in gamma counter.

Half-maximum inhibition of radioiodinated ligand binding by human recombinant CF6 was observed at 300 pg/tube. An appropriate amount of cold recombinant CF6 added to the RIA sample was precisely determined by the present RIA. The intra- and interassay coefficients of variance were 8.0% and 10.2%, respectively.

The immunoreactive substances present in the human urine were characterized by high-performance liquid chromatography (HPLC). The samples, treated with a Sep-Pak C18 cartridge, were applied on reverse-phase HPLC using an Inertsil ODS-2 C18 column (4.6 × 250 mm) (GL Science, Inc. Tokyo, Japan) and were followed by linear gradient of acetonitrile ranging from 20% to 60% over 30 minutes at a flow rate of 1 mL/min.

Synthesis of antibody for CF6

Synthetic CF6 fragment (human Cys-10-27 amino acid) solution was emulsified with an equal volume of Freund's complete adjuvant, and used for immunizing New Zealand white rabbits. The cross-reactivity of anti-CF6 antibody and the characterization of immunoreactive substances present in the human plasma were examined as previously.

Measurement of ADMA

Plasma ADMA level was measured by HPLC, as previously described [12]. Briefly, plasma (1 mL) was mixed with 2 mL of 10% trichloroacetic acid, put on ice for 10 minutes, and centrifuged at 2500g for 15 minutes. The resulting supernatant was evaporated in vacuo to dryness, and then loaded to a Bond Elut PRS column. After washing with 10 mL of 1 mol/L pyridine, ADMA was eluted by 10 mL of 3 mol/L ammonia and evaporated in vacuo to dryness. The extract was incubated with 20 μL phenylthiocarbamoyl solution (ethanol:triethylamine:water:phenyl isothiocyanate = 7:1:1:1, vol/vol) for 20 minutes at room temperature. The dried samples were applied on reverse-phase HPLC using...
a YMC-Pack ODS-AM column (YMC Co., Kyoto, Japan) and 60 mmol/L acetic buffer (pH 6.6)/0.05% TFA elution with a linear gradient of acetonitrile ranging from 6% to 60% over 25 minutes at 1 mL/min flow rate. Amounts of ADMA in the medium were estimated from a standard curve of synthetic ADMA.

Analysis of other variables

Serum sodium and potassium ion levels were measured with a flame photometer. Serum uric acid, glucose, albumin, calcium, phosphate, and creatinine levels and hemoglobin were measured by an autoanalyzer method. Plasma nitrate + nitrite (NOx) level was measured by the Griess method using NOx colorimetric assay kit (Cayman Chemical Co). Briefly, nitrate was converted to nitrite with cofactor and nitrate reductase, and total nitrite was measured at 540 nm absorbance by reaction with Griess reagent. Von Willebrand factor was measured by ristocetin agglutination method (SRL, Tokyo, Japan). Chemical Co). Brieﬂy, nitrate was converted to nitrite with cofactor and nitrate reductase, and total nitrite was measured at 540 nm absorbance by reaction with Griess reagent. Von Willebrand factor was measured by ristocetin agglutination method (SRL, Tokyo, Japan).

Statistics

Values are shown as mean ± one standard error (SE). Differences of mean values were assessed by a paired or unpaired Student t test for comparison of two variables and by an analysis of variance (ANOVA) for comparison for multiple variables. Relationships between two continuous variables were assessed by a regression analysis using the Pearson correlation coefficient. Differences in items were analyzed by chi-square test or Fisher’s exact probability test. A P value less than 0.05 was considered statistically significant.

RESULTS

Patient profiles

Table 1 shows the baseline characteristics of the patients with ESRD. Cyclooxygenase inhibitor was daily administered in ﬁve patients with ESRD. Hypertension was present in 79%, diabetes mellitus in 24%, and hyperlipidemia in 4%. Of all patients, 87 showed anuria.

Relationship between CF6 and cardiovascular events

Plasma CF6 level was 33.2 ± 0.9 ng/mL in patients with ESRD. Ninety-three (98%) patients had CF6 levels above the upper limit of the normal range (<16 ng/mL). The level in patients with chronic renal failure and without maintenance dialysis was 21.9 ± 1.3 ng/mL, and was higher than that in control subjects (12.8 ± 0.5 ng/mL) but lower than that in patients with dialysis (both P < 0.05). The plasma CF6 level was signiﬁcantly reduced after dialysis from 41.2 ± 4.2 ng/mL to 34.3 ± 2.4 ng/mL (N = 10, P < 0.05). By univariate analysis, plasma CF6 level was positively correlated with serum creatinine level (r = 0.36, P < 0.01) (Fig. 1), but was not correlated with duration of dialysis treatment and serum albumin level. There was no correlation between plasma CF6 level and Kt/V.

Urinary CF6 level was 112.6 ± 33.8 ng/mg creatinine in control subjects. In most patients with ESRD, urine was not excreted. In eight patients with urine excretion, urinary CF6 level was 192.7 ± 18.6 ng/mg creatinine, but the daily urine volume was <200 mL in all patients. Immunoreactive CF6 in the urine emerged at 5-minute fraction and was not identical to that of authentic human CF6 (22 minutes).

Sixty-six cardiovascular events were observed in 51 patients (Table 2). They included three myocardial infarctions, 11 strokes, 20 anginas, 14 congestive heart failures, 16 arrhythmias (mainly atrial ﬁbrillation), one peripheral

<table>
<thead>
<tr>
<th>Variable</th>
<th>Total population (N = 95)</th>
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<tbody>
<tr>
<td>Demographic</td>
<td>57.8 ± 13.7</td>
</tr>
<tr>
<td>Age years</td>
<td>52/43</td>
</tr>
<tr>
<td>Gender men/women</td>
<td></td>
</tr>
<tr>
<td>Cardiovascular risk factors</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>79%</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>24%</td>
</tr>
<tr>
<td>Hyperlipidemia</td>
<td>4%</td>
</tr>
<tr>
<td>Biochemical</td>
<td></td>
</tr>
<tr>
<td>Serum sodium mmol/L</td>
<td>140.1 ± 3.1</td>
</tr>
<tr>
<td>Serum potassium mmol/L</td>
<td>4.4 ± 0.7</td>
</tr>
<tr>
<td>Serum calcium mg/dL</td>
<td>9.7 ± 1.3</td>
</tr>
<tr>
<td>Serum phosphate mg/dL</td>
<td>5.0 ± 1.4</td>
</tr>
<tr>
<td>Serum glucose mg/dL</td>
<td>120 ± 60</td>
</tr>
<tr>
<td>Serum albumin g/dL</td>
<td>3.7 ± 0.4</td>
</tr>
<tr>
<td>Serum uric acid mg/dL</td>
<td>5.8 ± 1.3</td>
</tr>
<tr>
<td>Hemoglobin g/dL</td>
<td>10.4 ± 1.7</td>
</tr>
<tr>
<td>Low-density lipoprotein cholesterol mg/dL</td>
<td>100.0 ± 3.7</td>
</tr>
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Sixty-six cardiovascular events were observed in 51 patients (Table 2). They included three myocardial infarctions, 11 strokes, 20 anginas, 14 congestive heart failures, 16 arrhythmias (mainly atrial fibrillation), one peripheral
Table 2. Cardiovascular events in patients with end-stage renal disease (ESRD)

<table>
<thead>
<tr>
<th>Cardiovascular events</th>
<th>Number of patients (N D 95)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angina</td>
<td>20 (21%)</td>
</tr>
<tr>
<td>Arrhythmia</td>
<td>16 (17%)</td>
</tr>
<tr>
<td>Congestive heart failure</td>
<td>14 (15%)</td>
</tr>
<tr>
<td>Stroke</td>
<td>11 (12%)</td>
</tr>
<tr>
<td>Myocardial infarction</td>
<td>3 (3%)</td>
</tr>
<tr>
<td>Peripheral artery disease</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Retinal artery thrombosis</td>
<td>1 (1%)</td>
</tr>
<tr>
<td>Total</td>
<td>66 (69%)</td>
</tr>
</tbody>
</table>

artery disease, and one retinal artery thrombosis. Plasma CF6 level was higher in patients complicating ischemic heart disease (myocardial infarction and/or angina) than in those free of cardiovascular events (37.2 ± 1.9 ng/mL vs. 30.8 ± 1.3 ng/mL, P < 0.05) (Fig. 2).

Von Willebrand factor was positively correlated with plasma CF6 level in dialysis patients (N = 12) (r = 0.58, P < 0.05), whereas plasma NOx level was negatively correlated with plasma CF6 level (N = 16) (r = −0.51, P < 0.05).

Relationship between ADMA and cardiovascular events

Plasma ADMA level was 3.04 ± 0.38 µmol/L in patients with ESRD. All patients had ADMA level above the upper limit of the normal range (<1.16 µmol/L). By univariate analysis, plasma ADMA level was positively correlated with duration of dialysis treatment (r = 0.29, P < 0.02) (Fig. 3), but was not correlated with serum creatinine or albumin level. Plasma ADMA level was significantly higher in patients complicating either ischemic heart disease (4.34 ± 0.75 µmol/L) or arrhythmia (4.28 ± 0.90 µmol/L) than in those free of cardiovascular events (2.78 ± 0.15 µmol/L, both P < 0.05) (Fig. 4).

CF6, ADMA, and traditional risk factors in cardiovascular events

In a multiple regression model, plasma levels of CF6 (r = 0.237, P = 0.023) and ADMA (r = 0.256, P = 0.0226) were independently related to the occurrence of ischemic heart disease in patients with ESRD. However, none of traditional risk factors, such as hypertension, diabetes mellitus, and hyperlipidemia, was significantly related to the occurrence of ischemic heart disease in dialysis patients.

Relationship between CF6 and ADMA and its implications

As shown in Figure 5, plasma level of CF6 was positively correlated with that of ADMA in patients with ESRD (r = 0.25, P < 0.05).

The patients with higher plasma levels of CF6 (≥33 ng/mL) and ADMA (≥3 µmol/L) had a greater number of cardiovascular events compared to those with lower plasma levels of CF6 (<33 ng/mL) and ADMA (<3 µmol/L) [15 events in 20 patients (75%) vs. 13 events in 29 patients (45%), P < 0.05 by chi-square test]. The patients with higher plasma levels of both compounds also tended to have a greater number of cardiovascular events.
than those with higher ADMA level and lower CF6 level [11/22 (50%), P = 0.096] and those with lower ADMA level and higher CF6 level [11/24 (46%), P = 0.069].

DISCUSSION
The present study showed that plasma levels of CF6 and ADMA were threefold increased in patients with ESRD, that plasma levels of CF6 and ADMA were both higher in patients complicating ischemic heart disease (myocardial infarction and/or angina) than in those free of cardiovascular events by the cross-sectional retrospective observation, and that in a multiple regression model, plasma levels of CF6 and ADMA were independently related to the occurrence of ischemic heart disease.

CF6 as a cardiovascular risk factor in ESRD

Traditional cardiovascular risk factors, such as hypertension, hypercholesterolaemia, diabetes, and smoking, are often present in patients with ESRD. However, these cardiovascular risk factors only partly account for increased cardiovascular events in ESRD [1]. Prostacyclin exerts a number of profound effects, including inflammation, thrombogenesis, cell growth, and peripheral circulation [13], and its effect is widespread because prostacyclin receptor is localized in various sites such as heart, aorta, liver, kidney, and brain [14]. Endothelial dysfunction occurs before the onset of overt vascular disease, and the reduced generation of prostacyclin, which has an important role in the regulation of renal function in health and disease, might contribute to progression of cardiovascular disease. Indeed, we showed that plasma NOx level, a marker of endothelial function, was negatively correlated with plasma CF6 level, and von Willebrand factor, a marker of endothelial damage, was positively correlated with plasma CF6 in patients with ESRD.

The present study showed that plasma CF6 level was threefold higher in patients with ESRD, and was significantly higher in dialysis patients complicating ischemic heart disease than in those free of cardiovascular events by the cross-sectional retrospective observation. In a multiple regression model, plasma CF6 level was independently related to the occurrence of ischemic heart disease despite the fact that hypertension, diabetes mellitus, and hyperlipidemia were not significantly related to that of ischemic heart disease in ESRD. Thus, these findings seem to support the hypothesis that accumulation of CF6 may be a novel cardiovascular risk factor, especially for ischemic heart disease, in patients with ESRD. These also suggest that ESRD and atherosclerotic vascular disease could be independently associated with the increase in the plasma level of CF6.

It is unclear whether the higher level of CF6 may be related to the genesis of ischemic heart disease or secondary to ischemic heart disease in patients with ESRD. However, it is noted that in these patients, not only the plasma level of CF6 but also that of ADMA was remarkably elevated compared with healthy control individuals. In patients with higher plasma levels of ADMA (≥3 μmol/L) and CF6 (≥33 ng/mL), incidence of cardiovascular events tended to be greater than in those with higher level of ADMA and lower level of CF6 or those with lower level of ADMA and higher level of CF6. Thus, synergism of these compounds might play an important role in the occurrence of ischemic heart disease presumably via inhibition of prostacyclin and nitric oxide production. Plasma CF6 level was not correlated with Kt/V but was positively correlated with serum creatinine level. Dialysis treatment slightly but significantly reduced the plasma level of CF6. These suggest that modulation of serum creatinine level by dialysis treatment might be beneficial to prevent the occurrence of cardiovascular accidents.

The high level of plasma CF6 in patients with ESRD might be caused by no excretion or markedly reduced urine. This is indicated by our findings that plasma CF6 level was greater in patients with chronic renal failure without dialysis treatment than in control subjects, and was further increased in patients with ESRD. Indeed, metabolites of CF6 were excreted to the urine at ~100 ng/mg creatinine in control subject, whereas no excretion to the urine was found in most patients with ESRD. Another possible explanation for the high level of plasma CF6 is that the generation of CF6 is enhanced in patients with ESRD. We previously showed that shear stress stimulates the gene expression and release of CF6 in human vascular endothelial cells [15]. Thus, a hypertensive state in ESRD might be involved in the elevation of plasma CF6. Because CF6 is synthesized in an
immature form in the cytosol and accumulated within the mitochondria [16], tissue injury or apoptosis could enhance its release into the systemic circulation. Indeed, apoptosis was reported to be enhanced in aorta and left ventricle obtained from the animal models of ESRD [17].

ADMA as a cardiovascular risk factor in ESRD

The endothelium in patients with ESRD is dysfunctional, as suggested by findings of several clinical studies [18, 19], but the mechanism underlying this defect is not fully understood. Kari et al [18] found that endothelial dysfunction in uremic children was related to the plasma ADMA level. Hand, Haynes, and Webb [19] showed that endothelial dysfunction was reversed after hemodialysis sessions. Therefore, accumulation of ADMA might explain endothelial dysfunction in patients with ESRD. In the present study, we showed that plasma ADMA level was higher in patients complicating ischemic heart disease and/or arrhythmia than in those free of cardiovascular events by the cross-sectional retrospective observation. These findings may be consistent with those of the recent prospective study showing that accumulation of ADMA is an important risk factor for cardiovascular disease in chronic renal failure. Recently, in patients with chronic atrial fibrillation, plasma NOx level was reported to be decreased [20]. Our finding that the plasma ADMA level was elevated in patients complicating arrhythmia, mostly atrial fibrillation, may explain the pathogenesis of reduced nitric oxide generation in atrial fibrillation. This issue remains to be elucidated.

In individuals at high risk of cardiovascular disease or with overt atherosclerotic vascular disease, ADMA level is associated with degree of endothelial dysfunction and with the reduction in nitric oxide elaboration [21, 22]. Kielstein et al [5] observed that plasma ADMA level was higher in patients with ESRD and atherosclerotic vascular disease than in those without vascular complications. These investigators suggested that ESRD and atherosclerotic vascular disease could independently cause ADMA level to rise in patients with ESRD.

Relationship between CF6 and ADMA

It is unknown whether a crosstalk is present between CF6 and ADMA in ESRD. We recently found that plasma nitric oxide level is negatively correlated with plasma CF6 level in patients with essential hypertension. Therefore the lower plasma level of nitric oxide due to the higher plasma level of ADMA may increase CF6. Indeed, this study clearly showed that in patients with ESRD, plasma NOx level was negatively correlated with plasma CF6 level. Alternatively, the decrease in prostacyclin per se or its second messenger cyclic adenosine monophosphate (cAMP) may influence ADMA generation and degradation via modulating protein arginine methyltransferase type 1 and dimethylarginine dimethylaminohydrolase activities. In ESRD, not only the production of reactive oxygen species but also shear stress to vascular endothelial cells is enhanced, and both of them could increase the plasma levels of these inhibitory compounds. Therefore, it seems possible that CF6 and ADMA are both increased independently by the common stimuli despite of no crosstalk between them.

In humans, traditional risk factors such as hypertension [23], hypercholesterolemia [24], and diabetes mellitus [25] are associated with elevated ADMA level, which causes impaired endothelium-dependent vasodilation. The elevated ADMA may in turn increase the plasma level of CF6 via endothelial dysfunction. These suggest that traditional risk factors also may contribute to the higher plasma levels of ADMA and CF6.

CONCLUSION

This report shows that CF6 may be a novel cardiovascular risk factor in ESRD by the cross-sectional retrospective observation. In light of the current findings, it appears that CF6 is associated with the occurrence of ischemic heart disease in patients with ESRD. These data raise the possibility of a new explanation for a high cardiovascular risk in ESRD and pathologic role of CF6 may be expected to be growing. Since the data of the present study was derived from the cross-sectional retrospective observation, we need a prospective study to evaluate this issue more precisely.

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