A Cholesteryl Ester Transfer Protein Gene Mutation and Vascular Disease in Dialysis Patients

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Abstract. Among patients undergoing maintenance hemodialysis, a decreased high-density lipoprotein cholesterol (HDL-C) concentration is among the most common abnormalities of lipid metabolism and apparently is an independent risk factor for vascular disease. A common missense mutation of cholesteryl ester transfer protein gene, D442G (Asp 442 to Gly), increases HDL-C levels through the reduced activity of cholesteryl ester transfer from HDL to VLDL, but the mutation also may lead to reduced activity of reverse cholesterol transport. To investigate the effect of the D442G polymorphism on atherosclerotic complications in dialysis patients, the genotype and allele frequency of the polymorphism were determined in 414 unselected dialysis patients and 220 control subjects, and postprandial serum lipid levels were measured in the dialysis patients. A similar genotype distribution was found between hemodialysis patients and healthy control subjects, and in dialysis patients with and without vascular disease. Serum levels of total cholesterol and HDL-C did not differ between patients with and without the mutation and in patients with and without vascular disease. However, patients with sub-median HDL-C levels (&lt;45 mg/dl) had an independent odds ratio of 1.8 for vascular disease (95% confidence interval, 1.04 to 3.2; P &lt; 0.05). In this low-HDL-C subgroup, patients with the D442G mutation had a significantly higher prevalence of vascular disease than those with no mutation (54.5% versus 24.4%; P &lt; 0.05), and an independent odds ratio of 4.9 (95% confidence interval, 1.05 to 22.65; P &lt; 0.05). In conclusion, the D442G mutation is an independent risk factor for atherosclerotic complications in dialysis patients with HDL-C levels below 45 mg/dl.

In maintenance hemodialysis patients, a high risk for atherosclerotic vascular disease has been well documented (1,2), and cardiovascular disease is the major cause of mortality in this setting (3). Dyslipidemia has been proposed as a major reason for this increased prevalence of atherosclerotic complications, although other predisposing factors such as hypertension and disorders of calcium and phosphorus metabolism are frequently observed (4).

In many studies of subjects without renal failure, an inverse relationship has been reported between serum high-density lipoprotein cholesterol (HDL-C) concentration and atherosclerotic complications, including ischemic heart disease and cerebral vascular disease (5–7). In hemodialysis patients, a reduced concentration of HDL-C is one of the most commonly noted abnormalities of lipid metabolism (8,9). This form of dyslipidemia may arise from reduced activity of lipoprotein lipase, and/or lecithin-cholesterol acyltransferase (LCAT) and/or hepatic triglyceride lipase (9). As in patients without renal failure, HDL-C levels in dialysis patients are inversely related to occurrence of ischemic heart disease (4,8,10). These relationships may be explained in part by the role of HDL in reverse cholesterol transport, a process transferring cholesterol from peripheral tissues into HDL and then to the liver (11–13).

Recent studies have found that the missense mutation D442G (Asp442 to Gly) in exon 15 of the cholesteryl ester transfer protein (CETP) gene is common in the Japanese general population (14,15). CETP mediates the transfer of cholesteryl esters from HDL into triglyceride-rich lipoproteins, and thereby stimulates reverse cholesterol transport (16). Because the D442G mutation results in markedly increased HDL-C levels in homozygotes and moderate increases in heterozygotes (14,15), subjects with the mutation appear to be at lower risk for atherosclerotic complications. However, CETP involvement in reverse cholesterol transport suggests the opposite (16). Indeed, in a large population-based survey of Japanese-American men, subjects with CETP deficiency and HDL-C concentrations between 40 and 60 mg/dl were shown to have excessive occurrence of coronary heart disease compared to those without the deficiency (17). Hemodialysis patients have been reported to have decreased rates of cholesteryl ester transfer from HDL (13), which indicates impaired reverse cholesterol transport. Therefore, dialysis patients carrying the D442G mutation may be at greater risk for atherosclerotic...
complications, considering that the mutation would further reduce activity of reverse cholesterol transport, even though their mutation otherwise tends to lower their risk by increasing HDL-C concentration.

In the present study, we examined interrelationships between the prevalence of evident "large vessel" disease, the D442G mutation in the CETP gene, and HDL-C status in a large population of unselected hemodialysis patients using multivariate logistic regression analysis.

Materials and Methods

From July to December 1997, we investigated all subjects undergoing hemodialysis at the three dialysis centers in the Niigata and Fukui prefectures. The study subjects consisted of 414 Japanese hemodialysis patients (246 men, 168 women; age range, 20 to 88 yr; mean, 59.0). Hemodialysis had been initiated because of end-stage renal disease due to chronic glomerulonephritis (n = 245), diabetic nephropathy (n = 65), polycystic kidney disease (n = 21), Alport's syndrome (n = 2), nephrosclerosis (n = 37), chronic pyelonephritis (n = 15), toxemia of pregnancy (n = 6), a urogenital malformation or malignancy (n = 2), miscellaneous nephropathies (n = 6), and shrunken kidney of unknown etiology (n = 15). All patients were undergoing three weekly 4-h sessions of hemodialysis, generally using high-flux membranes and standard heparin doses for anticoagulation. The mean duration of hemodialysis treatment was 8.8 ± 7.5 yr. Informed consent for study participation was obtained from each subject. Smoking habits were defined on the basis of current cigarette smoking or previous habitual smoking. Hypertension was defined as a systolic BP >160 mmHg or a diastolic pressure >90 mmHg, or the use of antihypertensive drugs. Hemodialysis patients were given dietary advice that typically suggested a 30 to 35 kcal/kg. 1.2 g protein/kg diet (55 to 60% carbohydrate, 25% fat, and 15 to 20% protein).

Biochemical Assays

Venous blood samples (serum and whole blood) were collected 2 to 3 h after meals from all dialysis patients. Total serum cholesterol levels were measured by a standard enzymatic method. HDL-C was measured in the supernatant after precipitation of other lipoprotein fractions with phosphotungstic acid (18). Whole blood samples for genotyping of the D442G mutation were obtained postprandially from 220 healthy control subjects without renal disease or apparent vascular diseases (105 men, 115 women; age range, 16 to 79 yr; mean 53.1). The control subjects have normal levels of serum creatinine <1.2 mg/dl, a serum level of total cholesterol <250 mg/dl (range, 133 to 248 mg/dl; mean 183), and a serum level of HDL-C <100 mg/dl (range, 28 to 98 mg/dl; mean 59). They had no clinical sign of coronary artery disease, peripheral vascular disease, or cerebrovascular disease.

Assessment of Vascular Disease

To investigate the effect of CETP D442G mutation on vascular disease, we studied the prevalence of apparent vascular disease in the dialysis patients according to the following criteria.

Coronary Artery Disease. A previous myocardial infarction was defined as a heart attack diagnosed by a physician based on chest pain, electrocardiographic changes, and enzyme determinations or the results of coronary angiography. In patients with chest pain and ischemic electrocardiographic abnormalities, an exercise test or dipyridamole thallium scintigraphy was performed. Patients with a history of myocardial infarction or a positive result in one of these tests were diagnosed with coronary artery disease.

Peripheral Vascular Disease. Peripheral vascular disease was diagnosed by diminished pulses on clinical examination combined with comparison of ankle and arm systolic BP and/or peripheral angiography. An ankle/arm index <0.95 indicated the presence of peripheral vascular disease.

Cerebral Vascular Disease. Cerebral vascular disease was suspected on clinical grounds, i.e., rapidly developing signs of focal disturbance of cerebral function such as hemiparesis, hemisensory impairment, cerebellar ataxia, and vertigo with oculomotor nerve palsy. Stroke was diagnosed when clinical neurologic deficiency persisted for more than 24 h and transient ischemic attack was diagnosed when it resolved within 24 h. The diagnoses were confirmed by computed tomography or magnetic resonance imaging. Brain hemorrhage and subarachnoid hemorrhage were excluded.

A patient was considered to have a vascular disease when at least one of these three defined vascular diseases was present. Vascular disease was identified in 85 patients, while the remaining 329 had no apparent vascular disease.

Determination of the D442G Mutation in the CETP Gene

Genomic DNA was extracted from peripheral blood leukocytes using a commercially available kit (Collectagene; Takara Otsu, Shiga, Japan). Identification of the D442G mutation in the CETP gene was performed by PCR using a pair of primers described by Sakai and coworkers: 5'-TCATGAAACGCAAGGGCTAGCCCTCTCCG-3' and 5'-AGCCAACTGTTAGAGGCCCTTGTCTGT-3' (15). The amplification reaction mixture consisted of 1 µg of genomic DNA, 0.2 µM of each primer, 0.125 mM of each dNTP, 2.5 µl of a 10× buffer (100 mM of Tris-HCl at pH 8.3, 500 mM of KCl, and 15 mM MgCl2), and 1.25 U of Taq DNA polymerase (Takara Otsu) in a final volume of 25 µl. The reaction mixture was subjected to 35 cycles of 30 s at 94°C, 30 s at 61°C, and 45 s at 72°C. The final cycle was for 10 min at 72°C. Ten microliters of the 180-bp PCR product was subjected toMspI digestion (5 U in a 15-µl digest) at 37°C for 1 h. The digests were electrophoresed on 10% polyacrylamide gels. After electrophoresis, the gel was stained with ethidium bromide and visualized by ultraviolet transillumination.

Statistical Analyses

Analyses were conducted using the Statistical Package for Social Sciences software program. Continuous variables are expressed as mean ± SD. Differences in continuous variables between patients with and without vascular diseases and those between patients with and without the D442G mutation were assessed by a nonparametric test (Mann–Whitney U test). χ2 analysis was used to compare the distribution of genotypes and alleles of the D442G mutation between patients with and without vascular diseases, and to assess differences in noncontinuous variables between groups. Measures of association between the prevalence of vascular disease and the presence of the D442G mutation were assessed using odds ratios. Odds ratios were calculated using a logistic regression analysis and adjusted for covariates presented in the Results. P < 0.05 (two-tailed tests) was considered statistically significant.

Results

Among 414 hemodialysis patients, 27 subjects (6.5%) were heterozygous and no subject was homozygous for the D442G mutation. The 220 healthy control subjects included 18 het-
erozygotes (8.2%) and no homozygotes. No significant difference in genotype distribution was seen between dialysis patients and healthy control subjects. Compared with healthy control subjects, dialysis patients had significantly lower post-prandial levels of total cholesterol (183 ± 31 versus 160 ± 35, P < 0.01) and HDL-C (55 ± 16 versus 48 ± 18, P < 0.01).

When HDL levels in dialysis patients were stratified according to quartiles (HDL-C <35, 35 to 44, 45 to 54, and >55 mg/dl), prevalences of the mutation in the HDL-C strata were 4.0, 6.8, 6.7, and 8.3% respectively, appearing to increase with increasing HDL-C level, although not significantly.

No significant differences were observed in age, gender ratio, dialysis duration, prevalences of hypertension or diabetes mellitus, or smoking habits between patients with and without the mutation. Postprandial levels of total serum cholesterol and HDL-C and prevalence of antilipemic drug therapy were also similar in patients with and without the mutation (Table 1).

Twenty-three patients had experienced a myocardial infarction, and 18 patients had angina pectoris diagnosed by an exercise test or by scintigraphy. Forty-seven patients had cerebrovascular disease and 12 patients had peripheral vascular disease. Patients with vascular disease (n = 85) had a higher mean age, higher frequencies of hypertension and diabetes mellitus, or smoking habits than those without vascular disease (Table 2). No significant difference in postprandial levels of total cholesterol or HDL-C was observed between patients with and without vascular disease. In the latter subject group (n = 329), 25 patients had coronary artery disease, seven patients had peripheral vascular disease, and two patients had cerebral vascular disease.

Prevalence of antilipemic drug treatment was similar in patients with and without the mutation. The HDL-C strata were separated at the sample median. When HDL levels in dialysis patients were stratified according to high versus low high-density lipoprotein cholesterol (HDL-C) for groups of patients with and without the D442G mutation. The HDL strata were separated at the sample median.

### Table 1. Characteristics of patients with and without the D442G mutation

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with D442G Mutation (n = 27)</th>
<th>Patients without D442G Mutation (n = 387)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>60.9 ± 12.1</td>
<td>58.9 ± 12.8</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>20/7</td>
<td>226/161</td>
</tr>
<tr>
<td>Hemodialysis duration (yr)</td>
<td>9.3 ± 7.3</td>
<td>8.7 ± 7.5</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>157 ± 36</td>
<td>160 ± 35</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>50 ± 16</td>
<td>47 ± 18</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>23 (85.2)</td>
<td>283 (73.1)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>4 (14.8)</td>
<td>57 (14.7)</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>13 (48.1)</td>
<td>164 (42.4)</td>
</tr>
<tr>
<td>Prevalence of antilipemic drug use (%)</td>
<td>3 (11.1)</td>
<td>32 (8.3)</td>
</tr>
</tbody>
</table>

* HDL-C, high-density lipoprotein cholesterol. Numbers in parentheses indicate percentages.

### Table 2. Characteristics of patients with and without vascular disease

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patients with Vascular Disease (n = 85)</th>
<th>Patients without Vascular Disease (n = 329)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>67.6 ± 10.5*</td>
<td>56.8 ± 12.3</td>
</tr>
<tr>
<td>Gender (M/F)</td>
<td>54/31</td>
<td>192/137</td>
</tr>
<tr>
<td>Hemodialysis duration (yr)</td>
<td>5.1 ± 5.3*</td>
<td>9.8 ± 7.8</td>
</tr>
<tr>
<td>Serum cholesterol (mg/dl)</td>
<td>157 ± 39</td>
<td>161 ± 34</td>
</tr>
<tr>
<td>HDL-C (mg/dl)</td>
<td>45 ± 17</td>
<td>48 ± 18</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>73 (85.9)*</td>
<td>233 (70.8)</td>
</tr>
<tr>
<td>Diabetes mellitus (%)</td>
<td>33 (38.8)*</td>
<td>28 (8.5)</td>
</tr>
<tr>
<td>Smoking history (%)</td>
<td>41 (48.2)</td>
<td>136 (41.3)</td>
</tr>
<tr>
<td>Prevalence of antilipemic drug use (%)</td>
<td>11 (12.9)</td>
<td>24 (7.3)</td>
</tr>
</tbody>
</table>

* P < 0.01 versus patients without vascular disease. Numbers in parentheses indicate percentages.

To assess the influence of HDL-C on prevalence of vascular disease, the odds ratio (OR) was estimated after adjustment for age, hypertension, gender, smoking, diabetes mellitus, hemodialysis duration, total cholesterol, and the D442G mutation.
When HDL-C was treated as a continuous value, it failed to be predictive. However, introduction of an HDL-C below/above median term (below-median HDL-C assigned the value 1; above-median HDL-C assigned the value 0) indicated that HDL-C status was a prognostic factor (OR 1.82; confidence interval [CI], 1.04 to 3.22; \( P < 0.05 \)). In a model including all factors mentioned above, a multiple stepwise logistic analysis retained age (\( P < 0.001 \)), diabetes mellitus (\( P < 0.001 \)), smoking (\( P < 0.05 \)), hemodialysis duration (\( P < 0.05 \)), HDL-C status (\( P < 0.05 \)), and hypertension (\( P = 0.055 \)) as associated with vascular disease. Other variables, including the D442G mutation, failed to be predictive.

Because among patients with lower HDL-C levels (<45 mg/dl), subjects with the D442G mutation had a higher prevalence of vascular disease compared to those with the wild type (Figure 1), the OR of the mutation was estimated in the subgroup of patients with low HDL-C levels (\( n = 204 \)) (Table 3), demonstrating a significant association between prevalence of vascular disease and the mutation. With no adjustment for any covariate, the OR for association between the mutation and prevalence of vascular disease was 3.81 (\( P < 0.05 \)). Adjustment for traditional risk factors for atherosclerotic complications (see Table 3) increased the OR to 4.51 (\( P < 0.05 \)). Adjusting only for HDL-C produced an OR of 3.57, and adjusting for the conventional risk factors plus HDL-C produced the highest OR, 4.87 (\( P < 0.05 \)). A multiple stepwise logistic regression analysis using a model including the risk factors and HDL-C retained age (\( P < 0.001 \)), diabetes mellitus (\( P < 0.001 \)), smoking (\( P < 0.05 \)), hypertension (\( P < 0.05 \)), and the D442G mutation (\( P < 0.05 \)) as associated with vascular diseases.

### Discussion

We describe two major findings in this study of a large group of unselected hemodialysis patients over a broad age range. First, a low HDL-C level (<45 mg/dl) seemed to be an independent risk factor for vascular disease in these patients. Second, in dialysis patients with low HDL-C levels, the D442G mutation in the CETP gene was associated with increased prevalence of vascular disease, independently of traditional risk factors such as age, diabetes mellitus, hypertension, and smoking.

Because hemodialysis patients have a high prevalence of cerebral and peripheral vascular disease as well as coronary artery disease (1–3), we investigated the association between these atherosclerotic complications and clinical factors, especially HDL-C status and the D442G mutation. Our finding that hemodialysis patients with reduced HDL-C levels have an increased prevalence of vascular disease was consistent with previous observations of risk factors for coronary artery disease in dialysis patients (4,8,10). HDL-C has a central position in reverse cholesterol transport, which mobilizes cholesterol from peripheral tissues into HDL and then conveys it to the liver (11–13). HDL-C also has other antiatherogenic properties such as antioxidant effects (19,20). Thus, a decrease in HDL-C levels is assumed to accelerate the development of atherosclerotic complications in dialysis patients.

Hemodialysis patients have been reported to have lower fasting levels of HDL-C than healthy subjects (9,21), a finding paralleling ours, although the levels were measured postprandially in our study. The decrease in HDL-C levels in dialysis patients results from decreased production reflecting reduced activity of lipoprotein lipase (9,22) and the decreased formation of cholesteryl ester-rich HDL due to the reduced activity of LCAT (9). When serum lipid profiles are studied, fasting lipid levels are usually measured. In this study, however, we used postprandial lipid levels for the following reasons. First, it was very difficult to collect fasting blood samples from all patients because in our dialysis centers, about half of the patients undergo hemodialysis during the afternoon. Second, HDL-C and total cholesterol levels do not change much after meals (23). Third, hemodialysis was usually initiated 2 to 3 h after breakfast or lunch. Because postprandial levels of HDL-C were reported to be approximately 5 to 10% lower than fasting ones (23,24), the lipid data in this study may be slightly lower than those obtained after an overnight fast.

The most notable finding in the present study was that a partial CETP deficiency resulting from the D442G mutation was an independent risk factor for atherosclerotic complications in dialysis patients with HDL-C levels below the median, but not in those with higher HDL-C levels. In a recent report, Zhong and coworkers (17) showed that the prevalence of coronary heart disease in Japanese-American men with the D442G mutation and HDL-C levels between 40 and 60 mg/dl was more frequent than in subjects without the mutation. Our results confirm their previous findings in a high-risk group of hemodialysis patients. These influences of CETP polymorphism on risk could be explained by the role of CETP in reverse cholesterol transport. CETP promotes exchange of HDL cholesteryl ester for triglyceride in triglyceride-rich lipoproteins. Subsequent actions of hepatic lipase on triglyceride-rich HDL enhance formation of smaller HDL particles, which may be both optimal effectors of cellular cholesterol efflux and good substrates for the LCAT reaction (11,16).

### Table 3. Odds ratios (OR) and 95% confidence intervals (CI) for an association between vascular disease and the D442G mutation in renal patients with sub-median HDL-C levels

<table>
<thead>
<tr>
<th>Covariates</th>
<th>OR</th>
<th>95% CI</th>
<th>( P ) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>3.73</td>
<td>1.09 to 12.77</td>
<td>0.036</td>
</tr>
<tr>
<td>Risk factors*</td>
<td>4.35</td>
<td>1.05 to 22.6</td>
<td>0.046</td>
</tr>
<tr>
<td>HDL-C</td>
<td>3.54</td>
<td>1.03 to 12.21</td>
<td>0.045</td>
</tr>
<tr>
<td>Risk factors* + HDL-C</td>
<td>4.87</td>
<td>1.05 to 22.65</td>
<td>0.043</td>
</tr>
</tbody>
</table>

* Each horizontal row depicts a different model including the listed covariates as potential confounders.

* Age, gender, dialysis duration, serum total cholesterol, hypertension, diabetes mellitus, and smoking status.
relatively low CETP levels, although such concentrations were not measured in this study. Dialysis patients also show lower activity of hepatic lipase (9), a key enzyme promoting reverse cholesterol transport. It is possible that these dialysis patients with the mutation have lower reverse cholesterol transport activity and more susceptibility to atherosclerotic complications than those without the mutation. An antatherogenic effect of CETP such as we suggest in this study is supported by an animal study showing that overexpressing CETP in mice with severe hyperlipidemia inhibited development of early atherosclerotic lesions (25). Additionally, the lower prevalence of vascular disease in dialysis patients with both the mutation and HDL-C levels above 45 mg/dl could indicate that a mild-to-moderate increase in HDL particle number masks a qualitative defect in the ability of HDL to participate in reverse cholesterol transport. This modification of the impact of the mutation on cardiovascular disease by HDL levels was also found and discussed in the report of Zhong and coworkers (17).

The present report is the first to show an excess of vascular disease associated with the CETP D442G mutation in dialysis patients. However, this study is preliminary, it is cross-sectional in design, and it does not include patients with subclinical large-vessel disease. In addition, it does not examine the effects of antihypertensive drugs, especially angiotensin-converting enzyme inhibitors, on the prevalence of vascular disease. Therefore, a prospective study of a larger population of dialysis patients is required to verify an independent effect of the CETP D442G mutation on the development of macrovascular disease in this population.

In summary, the high risk of vascular diseases in hemodialysis patients may be explained partly by low HDL-C levels (<45 mg/dl). In dialysis patients with such reduced HDL-C levels, the D442G mutation in the CETP gene was associated with greater occurrence of vascular disease, whereas in those with normal or high HDL levels, the mutation had no apparent influence on vascular disease. These preliminary findings suggest that in combination with low HDL-C levels, genetic CETP deficiency may aggravate a defect in reverse cholesterol transport and increase susceptibility to atherosclerosis in patients with chronic renal failure.

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References


