Serum Paraoxonase and Arylesterase Activities in Hemodialysis Patients

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Cardiac death from atherosclerosis is common in hemodialysis patients. Human serum paraoxonase (PON1), an esterase, is associated with high-density lipoprotein and inhibits the susceptibility to oxidization of low-density lipoprotein (LDL). The PON1 genetic polymorphisms of 192 Gln/Arg and 55 Leu/Met in the amino acid sequence are partly involved in the PON1 enzyme activity. We investigated the PON1 enzyme activities for paraoxon (paraoxonase) and phenylacetate (arylesterase), and the two polymorphisms in 96 patients undergoing hemodialysis and in 136 normal controls. Both activities were significantly lower in the hemodialysis patients than in the controls (97 ± 43 vs 155 ± 57 μmol/min/I for paraoxonase, and 71 ± 20 vs 92 ± 22 mmol/min/I for arylesterase, respectively). There was no difference in the distribution of the two polymorphisms between patients and controls, and in every subgroup classified by the polymorphisms, both paraoxonase and arylesterase activities were lower in patients than in controls. This suggested that the enzyme activities of PON1 decreased in hemodialysis patients, independent of the genetic polymorphism. The decrease in PON1 enzyme activity in hemodialysis patients may modify a susceptibility to oxidization of LDL, which contributes to an acceleration of atherosclerosis. J Atheroscler Thromb, 2000; 7: 152-158.

Key words: LDL oxidation, Atherosclerosis, Renal failure

Introduction

Human serum paraoxonase (PON1) is an esterase that hydrolyzes aromatic carboxylic acid esters, organophosphates, and carbamates (1). PON1 associates with apolipoprotein (apo) A-I and apo J in high-density lipoprotein (HDL) (2), and has been shown to reduce the susceptibility of low-density lipoprotein (LDL) to lipid peroxidation (3-6). The serum activity of PON1 decreases in patients with a variety of diseases, such as myocardial infarction (7), familial hypercholesterolemia (8), diabetes mellitus (8-10) or uremia (11). The activity also varies by genetic basis among healthy individuals (1). This protein has two polymorphic sites which are Leu-Met at position 55 of the amino acid sequence (L/M), and Gin-Arg at position 192 (Q/R) (12). Both polymorphisms are involved in serum PON1 concentrations or paraoxonase activity which is measured using paraoxon as a substrate (12, 13). The activity in subjects with the RR genotype is higher than in those with QQ, and the activity with the LL genotype is higher than in those with MM. The activity in each heterozygote shows a middle level. However, it has also been shown that arylesterase activity, the hydrolysing activity of PON1 for phenylacetate, is independent of those gene polymorphisms (12). The Q/R polymorphism has been reported to be associated with coronary heart disease (CHD) (14, 15), although there have also been several contrasting studies (16-18).

Cardiac death is the most frequent cause of death in patients undergoing hemodialysis (19), and one important cardiac disease in such patients is myocardial infarction which results from atherogenesis (20). Oxidized LDL is a
major factor in the acceleration of atherosclerosis (21), and such abnormal lipoproteins may be implicated in the development of CHD in uremic patients (22). Schiavon et al. reported that paraoxonase activity was significantly reduced in uremic patients and suggested that altered HDL subfraction was a principal cause of the reduction, the higher frequency of alloenzyme B being another possibility (11). In that study, the frequency of the A and B allozymes, a genetic polymorphism, was estimated by a different response to NaCl. The allozyme is caused by the Q/R polymorphism which can be more accurately distinguished by genetic analysis. We therefore investigated whether the 192L/M polymorphism was associated with the decreased enzyme activity in hemodialysis patients, and we also investigated the 55Q/R polymorphism to analyze more completely the genetic influence on the activity. In addition, we measured not only paraoxonase activity but also arylesterase activity to determine whether the decrease in PON1 enzyme activity is specific for paraoxon.

We found a decrease in both paraoxonase and arylesterase activities in hemodialysis patients, which were independent of the genetic polymorphisms.

Materials and Methods

Subjects

Patients undergoing hemodialysis (n=96; male/female, 57/39) were recruited from the outpatient clinics of three hospitals between November 1997 and February 1998. The mean duration of hemodialysis was 3.9 years (0-22 years). The subjects had no history of cardiovascular disease during the 6 months prior to the study. Their clinical characteristics are shown in Table 1. CHD was defined as any one of the following: 1) acute myocardial infarction or confirmed non-acute myocardial infarction based on serial readings of baseline and biennial electrocardiograms; 2) coronary artery disease requiring bypass surgery or angioplasty; or 3) angina confirmed by angiography or by ischemic changes on non-invasive testing (23). Control subjects (n=136) who visited a medical center in Kochi prefecture for medical examination were recruited (Table 1). They were confirmed as being of normal status by physical and laboratory examinations including a normal resting electrocardiogram. They did not have familial hypercholesterolemia, diabetes mellitus (as confirmed by an oral glucose tolerance test), or a history of cardiovascular disease.

Sample collection

All blood samples were obtained from patients just prior to hemodialysis. To study the immediate effect of hemodialysis on paraoxonase activity, blood samples were obtained both before and after hemodialysis from 20 patients.

Paraoxonase and arylesterase activity and other biochemical analysis

The serum paraoxonase and arylesterase activity were measured using the method described by Eckerson et al. (24) with slight modification. Serum was preincubated with 5 µM eserine for 10 minutes at room temperature to inhibit serum butyrylcholinesterase activity. Paraoxonase activity was measured by adding serum to 1 ml Tris/HCl buffer (100 mM, pH 8.0) containing 1 mM CaCl$_2$ and 1 mM paraoxon (Sigma). The rate of generation of p-nitrophenol was determined at 412 nm, 25°C using a continuously recording spectrophotometer (Pharmacia LKB Biochrom 4060). Arylesterase activity was measured by adding serum to 1 ml Tris/HCl buffer (10 mM, pH 8.0) containing 1 mM CaCl$_2$ and 1 mM phenylacetate (Sigma). The rate of hydrolysis of phenylacetate was determined spectrophotometrically at 270 nm, 25°C. The plasma concentrations of total cholesterol and triglycerides were measured by enzymatic methods using an autoanalyzer. The plasma HDL-cholesterol (HDL-C) concentration was determined using a kit based on the dextran sulfate, phosphotungstate and magnesium precipitation method. The apolipoprotein A-I (apo A-I) and apo A-II concentrations were measured by turbidometric immunoassays (ApoA-I Auto 2, ApoA-II Auto 2; Daiichi Pure Chemicals, Tokyo, Japan).

PON1 gene polymorphisms

Genomic DNA was extracted from whole blood using a commercial kit (SMI test; Sumitomo, Tokyo, Japan). The two polymorphisms of PON1 were analysed using the polymerase chain reaction (PCR) and digestion of the amplified fragments with restriction enzymes as described previously (12). Primers for amplification of the polymorphic regions were 5’GAAAGTGTGATGTATAGCCCGCAG3’ and 5’T'TTAA--TCCAGAGCTAATGAAAAGCC3’ for 55L/M, and 5’TATTGTTGCTGTTGGGACC--TGAG3’ and 5’CACG-
CTAACCCAAATACATCTC3' for 192Q/R. DNA was denatured and then amplified for 35 cycles at 94°C for 30 s, 61°C for 30 s and 72°C for 30 s. The PCR product was digested with Hsp92II for L/M and Alwl for Q/R. The digested products were separated by 4% agarose gel and identified by ethidium bromide staining.

Statistical analysis
All values are presented as the mean ± standard deviation. Comparison of variables between two groups was performed using the unpaired t test. The values before and after hemodialysis were compared by the paired t test. Genotype frequencies were estimated by a chi-square test. All analysis including regression analysis was performed using a software program for a personal computer (JMP, ver 3.1, SAS Institute Inc. Cary, NC). p values <0.05 were considered significant.

Results
Paraoxonase and arylesterase activities
As shown in Fig. 1, paraoxonase activity was significantly lower in patients than in control subjects (97 ± 43 vs 155 ± 57 μmol/min/l, respectively). The arylesterase activity was also significantly lower in patients than in controls (71 ± 20 vs 92 ± 22 mmol/min/l). Since PON1 was associated with apo A-I on HDL, the PON1 mass may be accompanied by apo A-I mass. Therefore, the ratio of each activity divided by the HDL-C or apo A-I concentration was studied first. Paraoxonase activity (μmol/min/l) divided by either the HDL-C concentration (mg/dl) or apo A-I concentration (mg/dl) was not different between the patients and controls, while both the ratios of arylesterase activity (mmol/min/l)/HDL-C concentration (mg/dl) and arylesterase activity/apo A-I concentration (mg/dl) were higher in the patients. Subsequently, paraoxonase and arylesterase activities were compared with HDL components. Table 2 shows the relationships between paraoxonase or arylesterase activity and HDL-C, apo A-I or apo A-II concentration. Among the controls, paraoxonase activity showed a positive correlation with these three components, and arylesterase also showed a positive correlation to apo A-I and apo A-II. Among the patients, however, there were no relationships between those activities and HDL components except arylesterase activity vs apo A-II. The paraoxonase or arylesterase activity did not show any relationship to plasma total cholesterol or triglyceride concentration values. There were no differences in the ratio of HDL-C to apo A-I or to apo A-II between patients and controls, while the ratios of apo A-I to apo A-II concentration differed between them (4.7 ± 1.0 vs 4.4 ± 0.7, respectively, p < 0.02).

Since paraoxonase activity has been shown to decrease in patients with CHD or diabetes mellitus in reports, paraoxonase and arylesterase activities were compared between the hemodialysis patients with and

Fig. 1. Paraoxonase (PON) activity and arylesterase (ARYL) activity in hemodialysis patients and controls.
PON1 in Hemodialysis Patients

Table 2. Linear regression analysis between paraoxonase activity, or arylesterase activity and HDL-cholesterol, apo A-I or apo A-II concentration in hemodialysis patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>Paraoxonase activity</th>
<th>Arylesterase activity</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Patients</td>
<td>Controls</td>
</tr>
<tr>
<td>HDL-C</td>
<td>0.07</td>
<td>0.22 ± 0.6</td>
</tr>
<tr>
<td>Apo A-I</td>
<td>0.13</td>
<td>0.30 ± 0.8</td>
</tr>
<tr>
<td>Apo A-II</td>
<td>0.04</td>
<td>0.22 ± 0.6</td>
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*p < 0.05, *p < 0.02, **p < 0.001

without these diseases. There was no difference in paraoxonase or arylesterase activity between patients with and without diabetes. Paraoxonase activity did not differ between patients with and without CHD, although arylesterase activity was lower in non-diabetic patients with CHD than in those without (Table 3). There was also a significant difference in the activities between the non-diabetic patients without CHD and the controls (paraoxonase; 96 ± 43 vs 155 ± 57 μmol/min/l, respectively, p < 0.001 and arylesterase; 74 ± 18 vs 92 ± 22 mmol/min/l, respectively, p < 0.001).

Genetic polymorphism and enzyme activity

There was no difference in the frequency of genotypes classified by two genetic polymorphisms of PON1 between hemodialysis patients and controls (Table 4). The Q allele frequency of the Q/R polymorphism was 0.43 in patients and 0.41 in controls, and the L allele frequency of the L/M polymorphism was 0.94 and 0.94, respectively. Figure 2 shows the activity in each subgroup classified by two polymorphisms. Both paraoxonase and arylesterase activities were lower in every subgroup of patients compared with the same subgroup of controls.

Change of paraoxonase activity before and after hemodialysis

The activity after hemodialysis was significantly increased (97 ± 40 vs 124 ± 64 μmol/min/l, respectively, p < 0.001), but there was no difference in the value of the activity (μmol/min/l)/apo A-I concentration (mg/dl) after hemodialysis (1.21 ± 0.58 vs 1.07 ± 0.51, respectively).

Enzyme activity and other factors

Among the hemodialysis patients, the paraoxonase or arylesterase activity was not related to various factors of renal function, such as serum creatinine, blood urea nitrogen, uric acid and beta-2 microglobulin concentrations, as measured by linear regression analysis (data not shown). There was no relationship between each activity and hemodialysis duration or patient age. The paraoxonase and arylesterase activities among the subgroups classified by decades of age did not reveal any difference in the patient or control groups.

Discussion

We demonstrated that serum paraoxonase activity was significantly decreased in hemodialysis patients, which is consistent with previous studies (11, 25, 26). There was no difference in the ages of patients and controls, and there was no relationship between age and paraoxonase activity in any group (data not shown). In addition, we also found a decrease in arylesterase activity in patients, which may suggest that alteration of PON1 in uremic state

Table 3. Paraoxonase and arylesterase activities in hemodialysis patients with and without cardiovascular diseases.

<table>
<thead>
<tr>
<th></th>
<th>Patients with CHD</th>
<th>Patients without CHD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=6</td>
<td>n=18</td>
</tr>
<tr>
<td></td>
<td>DM</td>
<td>Non-DM</td>
</tr>
<tr>
<td></td>
<td>n=14</td>
<td>n=58</td>
</tr>
<tr>
<td>PON activity (μmol/min/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>104 ± 42</td>
<td>85 ± 40</td>
</tr>
<tr>
<td>Non-DM</td>
<td>64 ± 14</td>
<td>72 ± 22</td>
</tr>
<tr>
<td>ARYL activity (mmol/min/l)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DM</td>
<td>63 ± 23</td>
<td>74 ± 18</td>
</tr>
<tr>
<td>Non-DM</td>
<td></td>
<td></td>
</tr>
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</table>

*p < 0.05 vs non-diabetic patients without coronary heart disease.
n.s.: not significant.

Table 4. Frequencies (%) of the PON1 gene polymorphisms (192Q/R and 55L/M) in the hemodialysis patients and controls.

<table>
<thead>
<tr>
<th></th>
<th>patients (%)</th>
<th>controls (%)</th>
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<tbody>
<tr>
<td></td>
<td>QQ</td>
<td>QR</td>
</tr>
<tr>
<td>LQ</td>
<td>12.7</td>
<td>41.5</td>
</tr>
<tr>
<td>LM</td>
<td>6.4</td>
<td>6.4</td>
</tr>
<tr>
<td>MM</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>all</td>
<td>19.1</td>
<td>47.9</td>
</tr>
</tbody>
</table>

The P value for the 192Q/R polymorphism between patients and controls by the chi-square test is 0.29, and for 55L/M it is 0.94.
is not only an impairment of activity for a specific sub-
strate. Several mechanisms are possible for the decrease in 
PON1 enzyme activities in hemodialysis patients. The 
mechanism does not appear to be directly and immediate-
ly caused by hemodialysis, because paraoxonase activity 
following hemodialysis was relatively increased. This 
increase probably resulted from a blood concentration 
caused by hemodialysis, although the activity after 
hemodialysis was still lower when compared with that in 
the control subjects indicating that the decreased activity 
in patients was not simply caused by dilution of circulat-
ing blood.

It has been shown that paraoxonase activity is de-
creased in subjects with CHD (7) or diabetes mellitus (8-
10), and a certain percentage of the patients had these 
diseases in this study (25% and 21%, respectively). 
Therefore, we classified patients into subgroups based on 
the presence of these diseases and compared their 
paraoxonase and arylesterase activities with those in 
control subjects (Table 3). Both activities in each patient 
subgroup were lower than those in controls, indicating 
that the presence of these diseases was not a major 
cause of the decreased activity in patients.

Since Schiavon et al. (11) reported a higher frequency of 
alloenzyme B in uremic patients, we investigated PON1 
polymorphisms in patients and controls by genetic analy-
sis which could more precisely detect the genetic influ-
ence on PON1 enzyme activity. Both polymorphisms (55 
L/M and 192 Q/R) were involved in the paraoxonase 
activity, but not in the arylesterase activity, among 
patients as well as controls. Paraoxonase activity was 
higher in RR or LL homozygotes. However, the distribu-
tions of polymorphisms did not differ between patients and 
controls, and the activity in each haplotype of the two 
polymorphisms was lower in the hemodialysis patients 
than in the controls. Therefore, the two genetic polymor-
phisms are not causally related to the decrease in either 
paraoxonase or arylesterase activity in the hemodialysis 
patients.

In patients, each component of HDL (HDL-C, apo A-I 
and apo A-II concentration) was significantly lower than 
that in control subjects. Therefore, the decrease in 
paraoxonase and arylesterase activities was somewhat 
accompanied by a decrease in apo A-I mass, because the 
ratio of paraoxonase activity/apo A-I concentration in 
patients was comparable with that in controls, and aryles-
terase activity/apo A-I concentration was rather higher in 
patients. Although we did not measure PON1 mass in the 
subjects, it may be decreased by accompanying the 
reduced apo A-I, by accelerated catabolism or decreased 
production of PON1.

Some factors other than the decreased apo A-I mass 
could also affect the reduction of each activity. For 
example, metabolic change in the uremic milieu may 
modify the HDL composition or structure, which may 
possibly affect the active site of the enzyme resulting in a 
decrease in the activities. In the present study, both 
paraoxonase and arylesterase activities were significantly 
correlated with serum apo A-I concentrations in the

Fig. 2. Paraoxonase (PON) activity and arylesterase (ARYL) activity in subgroups classified by 55L/
M and 192Q/R polymorphisms of the paraoxonase gene in hemodialysis patients and controls.
controls, while these correlations were not detected among the patients. The ratios of apo A-I to apo A-II concentration also differed between patients and controls. This may be a sign of modified composition of HDL in hemodialysis patients. In the present study, there was no significant correlation of paraoxonase or arylesterase activity with several factors such as creatinine, beta-2 microglobulin etc., which were increased in uremic conditions. However, there still may be some circulating factors which inhibit enzyme activities, because small molecular matter is not completely removed by hemodialysis.

In conclusion, both serum paraoxonase and arylesterase activities are decreased in hemodialysis patients, independent of genetic polymorphisms. These changes may result in an acceleration of atherosclerosis in those patients. It is still unclear how to prevent the decrease in paraoxonase or arylesterase activity. Further investigations are required to clarify the causes of this decrease, and to find the means to prevent it.

References

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