Promoter Polymorphisms of Human Paraoxonase PON1 Gene and Serum Paraoxonase Activities and Concentrations

Ilia Leviev, Richard W. James

Abstract—Paraoxonase (PON) is a human serum enzyme entirely complexed to HDLs. Clinical interest in the enzyme was initially derived from its ability to neutralize highly toxic, exogenous derivatives (eg, pesticides and nerve gases). More recently, it has been hypothesized that PON fulfills an analogous role, that of protecting lipids from toxic oxidative modifications. Because the latter constitutes the principal atherogenic modification of serum LDLs, the ability of PON to limit oxidation represents a major antiatherogenic mechanism, and a growing body of data supports that contention. In particular, these data have demonstrated that the PON polymorphisms was analyzed by studying their association with serum concentrations and activities of PON. The study thus firmly establishes a genetic basis for variations in serum PON levels and, consequently, serum PON activity. It is consistent with the suggestion that variations in a major antioxidant function of high density lipoprotein are, to an important degree, genetically determined.

Key Words: HDL ■ atherosclerosis ■ oxidative stress ■ gene expression

Paraoxonase (PON) is a human serum enzyme entirely complexed to HDLs. Clinical interest in the enzyme was initially derived from its ability to neutralize highly toxic, exogenous derivatives (eg, pesticides and nerve gases). More recently, it has been hypothesized that PON fulfills an analogous role, that of protecting lipids from toxic oxidative modifications. Because the latter constitutes the principal atherogenic modification of serum LDLs, the ability of PON to limit oxidation represents a major antiatherogenic mechanism, and a growing body of data supports that contention.

In particular, these data have demonstrated that the PON content of HDL is a major determinant of the antioxidant function and, consequently, the anti-inflammatory capacity of the lipoprotein. Factors that influence PON levels are thus of major consideration for the efficacy of its protective function.

There are substantial interindividual variations in serum PON concentrations that cannot be fully explained by known, coding region polymorphisms. We have recently demonstrated an association of serum concentrations with a polymorphism at amino acid 54 of the coding region. The present study has identified 3 polymorphisms in the promoter region of the human PON1 gene. Cell transfection studies have revealed their variable impact on promoter activity, with up to 2-fold differences in reporter gene expression. Genotyping studies have established that the polymorphisms are frequent in the population, a finding that is consistent with a major impact on PON concentrations. The physiological relevance of the polymorphisms was underlined by showing that they are associated with highly significant differences in serum concentrations and activities of PON. The study thus firmly establishes a genetic basis for variations in serum PON levels and, consequently, serum PON activity. It is consistent with the suggestion that variations in a major antioxidant function of high density lipoprotein are, to an important degree, genetically determined.

Methods

Amplifying and Cloning the PON1 Gene Promoter Region

In initial studies, the DNA region upstream from the PON1 gene was amplified with the Genome Walker kit (Clontech) by using antisense primers (ATCCGGATCCGGGGATAGACAAAGGGATCGATG) and II (AGAGTGGCACGTCCTCCCCCATCCCAAGAG). By use of the Sslp library, a 1100-bp fragment was obtained. After sequencing this fragment, a third primer was designed (primer III, AAAACGCGTCCACCTCCACCATTGGGGAAAACCGTCAGATATTCCAGAAGAGAGAAG). By use of the primer II, a 107-bp fragment upstream from the PON1 gene coding region. Polymerase chain reaction (PCR) conditions were as follows: 94°C for 3 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 1 minute, and 72°C for 3 minutes. PCR products were digested with MluI and BglII (sites incorporated via the PCR primers) and cloned into Midl/BgII-digested plasmid. Cloned PCR products were sequenced by use of the Sequencing kit (Pharmacia).

Screening for PON Promoter Polymorphisms

Analysis of Position T(−107)C

Hybridization with allele-specific oligonucleotides was used to analyze polymorphisms at the T(−107)C position (Figure 2). PCR-
amplified fragments of the promoter region were loaded onto a nylon membrane (Porablot NYamp, Macherey-Nagel) and hybridized either with oligonucleotide IV (CCGCCGCCGCCCTCC), which is specific for nucleotide T at −107, or with oligonucleotide V (GGGGGAGGGGGGGGGG), which is specific for nucleotide C at −107. With oligonucleotide IV, hybridization was performed at 37°C, followed by 2 washes with 2× SSC/0.1% SDS at room temperature. With oligonucleotide V, hybridization was performed at 54°C, followed by 2 washes with 2× SSC/0.1% SDS at room temperature and 1 high-stringency wash with 1× SSC/0.1% SDS at 60°C.

Analysis of Position G(−824)A
Hybridization with allele-specific oligonucleotides was also used to analyze the polymorphism at position −824 (Figure 2). Allele-specific oligonucleotides were VIII (CAGCAGACAGCAGAGAAGAGA), which is specific for nucleotide C at −824, and VII (CTGAAGAAACAGCAGTCA), which is specific for nucleotide G at −824. Hybridization was performed at 44°C and was followed by 2 washes with 2× SSC/0.1% SDS at room temperature and 2 washes with 0.1× SSC/0.1% SDS at room temperature.

Analysis of Position G(−907)C
Allele-specific PCR was used to analyze this position. The sense allele-specific primers were VIII (CAGCAGACAGCAGACACAGAGAC), which is specific for nucleotide C at −907, and IX (CAGCAGACAGCAGACACAGAGAGAGAG), which is specific for nucleotide G at −907. The opposing antisense primer was primer I. PCR conditions were as described above, except that the annealing temperature was 62°C. Taq polymerase and Q solution from Qiagen were used in reactions.

For all oligonucleotides, hybridization was performed in ExpressHyb solution (Clontech) for 1.5 hours in the presence of 1 pmol/mL of 32P-labeled oligonucleotide.

**Figure 1.** Polymorphic sequences within the 5′ region of the PON1 gene. The polymorphic nucleotides are shown in bold type, and their positions are indicated with arrows. Numbering is determined by the PON initiation codon (to the right). Distances between the indicated nucleotide sequences are given in italics. The possible binding site for the transcription factor Sp1 is framed.
nucleotide positions

amplified DNA upstream from the PON open reading frame was sequenced and revealed 3 polymorphic sites at positions.

concentrations, samples with low, medium, and high concentrations of PON were selected on the rationale that the concentrations level may reflect differences in gene expressions. Amplified DNA upstream from the PON open reading frame was sequenced and revealed 3 polymorphic sites at nucleotide positions −107 (C or T), −824 (A or G), and −907 (C or G) (Figure 1). The numbering is with respect to the A of the PON initiation codon, which was assigned the value 0. The complete sequence of the region is accessible at Genbank, accession number AF051133. No other polymorphisms were detected in 100 samples analyzed by sequencing. Genotyping procedures were developed to analyze the promoter polymorphisms in the whole population (Figure 2). The distributions of the genotypes for the 3 promoter polymorphisms are shown in Table 1. In preliminary studies, genotype frequencies were compared in subjects aged <40 years (n=124) and >40 years (n=250). These were not found to be significantly different. The analysis revealed a strong association between the 3 polymorphisms (for −107×−907, χ²=185.5, P<0.0001; for −107×−824, χ²=133.5, P<0.0001; and for −824×−907, χ²=97.8, P<0.0001). A strong association was also observed between the promoter polymorphisms and the polymorphism influencing amino acid 54 of the PON1 gene coding region (for −107, χ²=31.7, P<0.0001; for −824, χ²=21.7, P<0.0005; and for −907, χ²=39.9, P<0.0001) but not with polymorphism 191.

**Promoter Polymorphisms Are Associated With Modulated Gene Expression**

The influence of the polymorphisms on gene expression was examined by using reporter gene constructs. These indicated strong independent impacts of 2 polymorphisms on transcriptional activity of the PON1 promoter (Figure 3). The −107C variant had 2 times higher activity than did the −107T variant (P<0.05), whereas the −824A polymorphism was 1.7 times more active than the −824G polymorphism (Figure 3B, P<0.05). This is in agreement with the observed higher serum activities and concentrations of PON associated with the −107C and −824A polymorphisms (see below). No significant differences in gene expression were noted when the −907C and −907G variants were analyzed (Figure 3C). Further studies are necessary to determine whether there are interactions between promoter polymorphisms with respect to gene expression and to define what other factors, both genetic and nongenetic, influence serum concentrations of PON. A

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**Results**

**Polymorphisms Exist in Promoter Region of Human PON1 Gene**

From an initial sample of 100 subjects, which had been characterized with respect to serum PON activities and concentrations, samples with low, medium, and high concentrations of PON were selected on the rationale that the concentration level may reflect differences in gene expression. Amplified DNA upstream from the PON open reading frame was sequenced and revealed 3 polymorphic sites at nucleotide positions −107 (C or T), −824 (A or G), and −907 (C or G) (Figure 1). The numbering is with respect to the A of the PON initiation codon, which was assigned the value 0. The complete sequence of the region is accessible at Genbank, accession number AF051133. No other polymorphisms were detected in 100 samples analyzed by sequencing. Genotyping procedures were developed to analyze the promoter polymorphisms in the whole population (Figure 2). The distributions of the genotypes for the 3 promoter polymorphisms are shown in Table 1. In preliminary studies, genotype frequencies were compared in subjects aged <40 years (n=124) and >40 years (n=250). These were not found to be significantly different. The analysis revealed a strong association between the 3 polymorphisms (for −107×−907, χ²=185.5, P<0.0001; for −107×−824, χ²=133.5, P<0.0001; and for −824×−907, χ²=97.8, P<0.0001). A strong association was also observed between the promoter polymorphisms and the polymorphism influencing amino acid 54 of the PON1 gene coding region (for −107, χ²=31.7, P<0.0001; for −824, χ²=21.7, P<0.0005; and for −907, χ²=39.9, P<0.0001) but not with polymorphism 191.

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**TABLE 1. Genotype Frequencies of PON Promoter Polymorphisms**

<table>
<thead>
<tr>
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</thead>
<tbody>
<tr>
<td>CC</td>
<td>0.36</td>
<td>0.57</td>
<td>0.32</td>
<td>0.42</td>
<td>0.46</td>
</tr>
<tr>
<td>CG</td>
<td>0.46</td>
<td>0.36</td>
<td>0.44</td>
<td>0.46</td>
<td>0.45</td>
</tr>
<tr>
<td>GG</td>
<td>0.18</td>
<td>0.07</td>
<td>0.24</td>
<td>0.11</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>G(−907)C</th>
<th>CC</th>
<th>CT</th>
<th>TT</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC</td>
<td>1</td>
<td>18</td>
<td>114</td>
<td>133</td>
</tr>
<tr>
<td>CG</td>
<td>23</td>
<td>145</td>
<td>2</td>
<td>170</td>
</tr>
<tr>
<td>GG</td>
<td>64</td>
<td>1</td>
<td>0</td>
<td>65</td>
</tr>
<tr>
<td>Total</td>
<td>88</td>
<td>164</td>
<td>116</td>
<td>368</td>
</tr>
</tbody>
</table>

Genotypes were determined in healthy volunteers (n=368 for −907, n=351 for −824, and n=374 for −107). At the top, numbers in parentheses correspond to the fractional distribution of each genotype. At the bottom, numbers correspond to subjects with the indicated genotype. Promoter polymorphisms are numbered from the A of the PON initiation codon, which was assigned the value 0. Coding region polymorphisms (L54M and Q191R) are numbered according to the amino acid positions.
minimum conclusion from these studies is that the promoter polymorphisms strongly influence gene expression.

**Promoter Polymorphisms Are Associated With Variations in Serum PON Concentrations and Activities**

Table 2 shows the PON concentrations and activities (with the nondiscriminatory substrate phenylacetate) as a function of the different polymorphisms. There were highly significant variations in serum concentrations and activities of PON as a function of all 3 promoter polymorphisms. Thus, −107T, −824G, and −907C were associated with lower serum PON levels, whereas −107C, −824A, and −907G were correlated with the highest concentrations and activities. A gene dose effect is also evident, with heterozygotes having intermediate values. Given the strong association between the promoter polymorphisms and the data on reporter gene expression, it remains to be determined to what extent each polymorphic site contributes to variations in enzyme levels (see below).

Table 3 shows the results of stepwise multiple regression analysis of the different parameters that could influence serum concentrations of PON. Model A was limited to PON1 gene polymorphisms. It shows that the T(−107)C polymorphism has a predominant effect, accounting for 24.7% of the variation in serum concentrations of PON. The G(−907)C polymorphism was also implicated but did not reach statistical significance (P=0.07). Interestingly, the coding region polymorphism affecting amino acid 54 also made a significant contribution to variations in serum PON concentrations (4.4% of the variation), whereas no significant contribution was observed for the coding region Q191R polymorphism.

The PON1 gene polymorphisms accounted for 29.1% of the variations in serum PON levels. Model B introduced other parameters, notably serum lipids, into the analysis. They did not modulate the associations of the T(−107)C and L54M mutations with serum PON, but in this model, the association with the G(−907)C polymorphism was also significant. In addition, HDL cholesterol, triglycerides, sex, and age were independently associated with variations in serum concentrations of PON. Model B accounted for 37.7% of the variation in serum PON levels.

**Discussion**

The present study has identified 3 common polymorphisms in the promoter region of the human *PON1* gene. Transfection studies demonstrated that they have an important impact on promoter activity, with up to 2-fold differences in reporter gene expression between polymorphisms. The physiological relevance of the polymorphisms was highlighted by showing that they are associated with highly significant differences in serum concentrations and enzymatic activities of PON. The study thus firmly establishes a genetic basis for variations in serum PON levels and, consequently, serum PON activity. The physiological aspects of these polymorphisms are important. It has recently been established that the PON content of HDL is a major determinant of the antioxidant capacity of the lipoprotein.5,7,8 Our results suggest that this antioxidant function of HDL is, at least in part, genetically determined.

The T(−107)C polymorphic site appears to have a dominant effect on expression of the *PON1* gene, with a minor contribution from the G(−907)C polymorphic site. We are presently examining the promoter sequence for possible transcription sites. The polymorphic position T(−107)C lies within the GGCGGG (the polymorphic site is italicized) consensus sequence of the binding site for the transcription factor Sp1.12 Mutations within this site have been previously shown to affect the promoter activity of other sites.13,14

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**TABLE 2. PON Mass Concentrations and Enzymatic Activities as a Function of Promoter and Coding Region Mutations**

<table>
<thead>
<tr>
<th></th>
<th>G(−907)C</th>
<th>G(−824)A</th>
<th>T(−107)C</th>
<th>L54M</th>
<th>Q191R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mass concentration, µg/mL</td>
<td>84.1 ± 20.8</td>
<td>90.8 ± 23.1</td>
<td>81.8 ± 19.9</td>
<td>106.9 ± 22.5</td>
<td>AA: 95.1 ± 24.6</td>
</tr>
<tr>
<td>Enzymatic activity, U/mL</td>
<td>77.5 ± 25.4</td>
<td>89.7 ± 23.4</td>
<td>84.7 ± 26.1</td>
<td>80.7 ± 27.8</td>
<td>85.7 ± 28.6</td>
</tr>
<tr>
<td>P</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are mean ± SD for serum PON mass concentrations and enzymatic activities with phenylacetate. Values of P are by ANOVA.
TABLE 3. Parameters Associated With Variations in Serum Concentrations of PON

<table>
<thead>
<tr>
<th>Model A parameter</th>
<th>Variation, %</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Polymorphism T(107)C</td>
<td>24.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Polymorphism L54M</td>
<td>4.4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Polymorphism G(907)C</td>
<td>1.0</td>
<td>0.07</td>
</tr>
<tr>
<td>Model B parameter</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Polymorphism T(107)C</td>
<td>24.7</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Polymorphism L54M</td>
<td>4.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>3.9</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>3.6</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Polymorphism G(907)C</td>
<td>1.2</td>
<td>0.04</td>
</tr>
<tr>
<td>Age</td>
<td>0.8</td>
<td>0.047</td>
</tr>
<tr>
<td>Sex</td>
<td>0.8</td>
<td>0.049</td>
</tr>
</tbody>
</table>

Stepwise forward multiple regression analysis of parameters associated with variations in serum concentrations of PON is shown. For model A, included in the analysis were polymorphisms G(–824)A and Q191R. The adjusted $r^2$ value for the model was 37.8. For model B, included in the analysis were polymorphisms G(–824)A and Q191R and cholesterol. The adjusted $r^2$ value for the model was 39.1.

In a previous study, we reported a significant association between the coding region L54M mutation and serum PON levels.9 The present study clarifies this point. It is, in part, due to the strong association with the promoter polymorphisms. However, this link does not explain completely the association of L54M with serum PON concentrations, which remained significant in the model containing the promoter polymorphisms. Thus, when we analyzed samples from subjects with the same T(107)C genotype (eg, TT homozygotes), there remained a significant difference in PON concentrations between subjects who were homozygous LL (88.5 ± 21.2 mg/mL, n = 23) or MM (78.2 ± 17.5 mg/mL, n = 33; by ANOVA, $P < 0.05$) for the L54M mutation. The observation is intriguing because the methionine-leucine difference at position 54 represents a conservative amino acid exchange. One possibility is that the latter could nevertheless affect either protein stability or the association of PON with HDL, the serum transport vector for the enzyme.

A second important implication of the present study relates to the association of the PON1 gene with the risk of vascular disease. Several studies have identified PON as an independent genetic risk factor for coronary disease,9,15–20 although there is no complete agreement on this point.21,22 These studies relate to the coding region polymorphisms L54M and Q191R. The ability of the promoter polymorphisms to modulate serum concentrations of the enzymes to an important degree suggests that they could influence the association between the coding region mutations of the PON1 gene and risk of disease. We are presently investigating this hypothesis. In this respect, it should be noted that polymorphisms affecting the Sp1 site have previously been shown to have particularly marked clinical consequences.23,24 A second possibility is that the promoter polymorphisms could be a confounding factor in the discordant results concerning the role of PON as a genetic risk factor. We are also studying this possibility.

Finally, it should be emphasized that observations involving PON also have implications in the toxicology field. Studies in animal models have clearly demonstrated that serum PON activity is protective against certain environmental poisons derived from organophosphates.2,25,26 In humans, the activity of the polymorphism arising from the Q191R coding region mutation undoubtedly has a major impact on susceptibility to these compounds. However, within subjects homozygous for Q191R alleles, there remains a considerable range of enzymatic activities,27,28 which will modulate susceptibility to poisoning. The present study indicates that in this case there is also a strong genetic influence.

Acknowledgments

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References


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