Polymorphisms in the human paraoxonase (PON1) promoter

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Paraoxonase (PON1) is a protein component of high-density lipoprotein (HDL) particles that protects against oxidative damage to both low-density lipoprotein and HDL and detoxifies organophosphorus pesticides and nerve agents. A wide range of expression levels of PON1 among individuals has been observed. We examined the promoter region of PON1 for genetic factors that might affect PON1 activity levels. We conducted a deletion analysis of the PON1 promoter region in transient transfection assays and found that cell-type specific promoter elements for liver and kidney are present in the first 200 bp upstream of the coding sequence. Sequence analysis of DNA from a BAC clone and a YAC clone identified five polymorphisms in the first 1000 bases upstream of the coding region at positions −108, −126, −162, −832 and −909. Additionally, the promoter sequences of two individuals expressing high levels of PON1 and two individuals expressing low levels of PON1 were analysed. The two polymorphisms at −126 and −832 had no apparent effect on expression level in the reporter gene assay. The polymorphisms at position −909, −162 (a potential NF-I transcription factor binding site) and −108 (a potential SP1 binding site) each have approximately a two-fold effect on expression level. The expression level effects of the three polymorphisms appear not to be strictly additive and may depend on context effects.

Keywords: PON1, promoter polymorphism, atherosclerosis, gene expression, organophosphorus insecticides

Introduction

Paraoxonase (PON1) is tightly associated with high-density lipoprotein (HDL) particles. Recent evidence shows that PON1 protects both low-density lipoprotein and HDL from oxidation (Mackness et al., 1991, 1993; Watson et al., 1995; Mackness et al., 1997; Aviram et al., 1998; Mackness et al., 1998a), a major step in the progression of atherosclerosis and heart disease. PON1 also protects against cholinesterase inhibition by the bioactive forms of organophosphorus pesticides such as diazinon (Li et al., 2000) and chlorpyriños (Li et al., 1993; Li et al., 1995; Shih et al., 1996; Li et al., 2000). PON1 also hydrolyses the nerve agents soman and sarin (Davies et al., 1996). Individuals show large variation in the expression levels of PON1 (La Du et al., 1986; Furlong et al., 1989; Davies et al., 1996; Richter & Furlong, 1999).

Two polymorphisms have been identified in the coding sequence of human PON1, L55M and Q192R. PON1L55 is correlated with higher PON1 activity and mRNA levels than PON1M55 (Blatter Garin et al., 1997; Leviev et al., 1997; Mackness et al., 1998b). However, there is considerable variation among individuals with some PON1M55 individuals having greater PON1 levels than some PON1L55 individuals (Brophy et al., 2000). The position 192 polymorphism results in substrate-dependent differences in the kinetics of hydrolysis of various substrates (Davies et al., 1996; Li et al., 2000). PON1Q192 has a higher $V_{max}$ for diazoxon and sarin hydrolysis than PON1R192 whereas the opposite is true for the substrate paraoxon (Li et al., 2000). Additionally, within each genotype, there is a great deal of variation in PON1 expression and activity level.
While the L55M polymorphism is associated with this variability, it is not likely to be the source.

Experiments on the relationship between PON1 levels and resistance to the oxon forms of diazinon and chlorpyrifos have clearly demonstrated that high PON1 levels are protective while low PON1 levels result in sensitivity. PON1 knockout mice (missing both plasma and liver PON1) are five- to 10-fold more sensitive to cholinesterase inhibition by diazoxon or chlorpyrifos oxon than wild-type mice (Shih et al., 1996; Furlong et al., 1998; Li et al., 2000). Reconstitution of the plasma PON1 in PON1 null mice by intraperitoneal injection of human PON1 reconstitutes resistance to diazoxon and chlorpyrifos oxon (Li et al., 2000). Our recent studies on the relationship between PON1 status (genotype and phenotype) and carotid artery disease have shown that PON1 levels are lower in patients homozygous for PON1*Q192 or heterozygotes compared to matched controls (Jarvik et al., 2000).

Since variation in PON1 levels has been shown to be physiologically significant, we examined the 5′ regulatory region for genetic factors responsible for the large observed inter-individual differences in levels. Recently, two groups (Leviev & James, 2000; Suehiro et al., 2000) reported on their analyses of the PON1 promoter region. Leviev and James found three polymorphisms at −107, −824 and −907 relative to the start codon. Suehiro et al. found two additional polymorphisms at −126 and −160 and confirmed the one at −107 (−108 in their report). Two of the polymorphisms, −107/108 and −824, were found to have effects on PON1 expression levels. We independently identified the five polymorphisms and report additional data on the effect of the polymorphisms on PON1 expression.

**Materials and methods**

**DNA sources**

PON1 promoter sequences came from three sources: a BAC clone (GS1-155M11, Genbank accession no. AC004022), a YAC clone (A97E9; Green et al., 1995; Clendenning et al., 1996), and DNA from volunteers who were participants in an Epidemiology Research and Information Center project at the Puget Sound Veterans Affairs Health Care System (PSVAHCS). The study was approved by both the University of Washington and the PSVAHCS human subject review processes. Subjects gave their written informed consent. Subject DNA was prepared from buflly coat preparations by a modification of the procedure of Miller et al. (1988) using Puregene reagents (Gentra, Minneapolis, MN, USA). The YAC DNA was prepared by a standard method (Hoffman & Winston, 1987).

**Cloning**

Regions 5′ to the PON1 gene were cloned by polymerase chain reaction (PCR). A 5.3 kb region was amplified from the YAC clone using the Extend PCR kit (Roche Molecular Biochemicals, Indianapolis, IN, USA) and the primers: CCCTAGGTACCCCGAG GAATTCGTAATAC and GTAATCTGAGTCGGGGA TAGACAAAG. The PCR product was first cloned using the Topo-XL kit (Invitrogen, Carlsbad, CA, USA), then cloned into pGL3-Basic (Promega, Madison, WI, USA) using the restriction sites KpnI and XhoI. The mammalian expression vector pGL3-Basic contains a multiple cloning site upstream of a firefly luciferase gene that has no promoter. A 6.2 kb region was amplified from the YAC clone using the TaqPlus Long kit (Stratagene, La Jolla, CA, USA). TOPO-XL cloned then transferred 5′ to the 5.3 kb promoter to generate an 11.5 kb region upstream of the PON1 ATG (primers CTTATGGTACCTTGTACCCCGTGA CAGTT and GCAGCACATGGATCTTAAATGTTCC). A 960 bp region was amplified from the YAC clone using Taq polymerase (Promega) and the primers CCCTAGGTACCGTGAAGGAAGACGACATGGGC and GTAATCTGAGTCGGGGA TAGACAAAG. The product was Topo-TA (Invitrogen) cloned and transferred to pGL3-Basic using XhoI and MluI. The 200 bp fragment was generated by SacI digestion of the 960 bp fragment and the other fragments were generated by ExonucleaseIII digestion (Life Technologies/Gibco BRL, Rockville, MD, USA) of the 5.3 kb region by a standard protocol (Beckler, 1996). Promoter regions of 960 bp were amplified from the volunteers and cloned as described above. The haplotypes discussed here are listed in Table 1. The QuikChange (Stratagene) site-directed mutagenesis system was employed to generate the BAC haplotype from haplotype C1/C2 for testing in the transient transfection system. Additionally, haplotypes E and G were generated by site-directed mutagenesis from the YAC haplotype while haplotype F was generated from the BAC haplotype. Cycle sequencing was used to verify that only the intended changes had been introduced.

**Sequencing**

The PON1 5′ regions were sequenced after cloning using Amplitaq FS or BigDye (PE Biosystems, Foster City, CA, USA) according to the manufacturer’s instructions. Sequence analysis and alignment used the online Biology Workbench software (NCSA, http://biology.ncsa.uiuc.edu/) and CLUSTALW (Higgins et al., 1992; Thompson et al., 1994). Clones were sequenced at least twice to ensure quality of the
data. The sequences of the regions subcloned from the YAC clone agreed with the published sequence (BAC GS1-155M11, Genbank accession no. AC004022) except at five positions: \(-C108T, -G126C, -G162A, -G832A, \) and \(-C909G where the base immediately preceding the ATG is \(-1\). These five polymorphisms were found in two separate subclones of the YAC clone, confirming that they were not errors introduced by PCR. For the haplotypes generated from the volunteers, genotypes were verified by identifying multiple clones with the same haplotype and sequencing the PCR products directly.

**Transient transfection**

The pGL3 plasmids containing the various PON1 promoter regions were transiently transfected into the human kidney cell line 293 (Graham et al., 1977) and the human hepatoma cell line HepG2 (Knowles et al., 1980). Transfection into 293 cells used 10 \(\mu\)l of lipofectin (Life Technologies) and 0.5 pmol of plasmid DNA. Transfection into HepG2 cells used 15 \(\mu\)l of lipofectin and 0.5 pmol of plasmid. Control plasmid pRL/CMV (50 ng) containing the Renilla luciferase driven by the CMV promoter was cotransfected with the promoter/reporter construct being tested. After 24 h, the lipofectin and DNA were removed by culture medium replacement and the cells were allowed to grow an additional 24 h in normal media before being lysed and analysed using the Dual-Luciferase Assay System (Promega). Data were normalized for transfection efficiency by the Renilla luciferase activity and by total protein concentration. Luciferase activities were determined on a Turner TD-20/20 Luminometer (Sunnyvale, CA, USA). Determinations of \(P\)-values were determined by \(t\)-test.

**PON1 status determination**

PON1\(_{192}\) and PON1\(_{55}\) genotypes were determined using PCR techniques and AlwI and NlaIII restriction enzyme analysis (Humbert et al., 1993). PON1 paraoxon and diazoxon hydrolysis rates were measured spectrophotometrically with lithium heparin plasma, as described (Davies et al., 1996; Richter & Furlong, 1999). A two-dimensional plot of diazoxon hydrolysis activities versus paraoxon hydrolysis activities provided individual PON1 phenotypes and an accurate confirmation of PON1\(_{192}\) genotype.

**Results**

We began our analysis by asking where the important promoter elements are in the region 5’ to the PON1 gene. Computer analysis (Schug & Overton, 1997) of the sequence immediately upstream of the
gene revealed that PON1 contains neither a canonical TATA nor a CAAT box and the region is GC-rich, typical of TATA-less promoters. We used deletion analysis to locate important regions in the PON1 upstream sequence. A YAC clone (A97E9) was identified that contained the region 5' to the PON1 coding sequence (Clendenning et al., 1996). Using PCR and standard cloning techniques, plasmids were generated containing 200, 960, 5294 bp, or 11.5 kb of sequence directly 5' to the PON1 gene. These sequences were cloned into pGL3-Basic (Promega), 5' to the reporter gene firefly luciferase. Additional plasmids were generated by Exonuclease III digestion from the 5' end of the 5294 bp fragment, providing plasmids with promoter regions of varying lengths.

Studies in mice (Primo-Parmo et al., 1996) and rabbits (Hassett et al., 1991) have demonstrated that PON1 is most highly expressed in the liver and HepG2 human liver cells have previously been shown to express PON1 (Navab et al., 1997; Feingold et al., 1998). In mouse kidney, PON1 is expressed at approximately half the level that occurs in liver (Primo-Parmo et al., 1996). Thus, 293 human kidney cells were employed to identify the location of cell-type specific elements. Each plasmid was cotransfected into the liver HepG2 cells with the control plasmid pRL-CMV (Promega). A subset of the plasmids was similarly transfected into the 293 kidney cell line. Figure 1 shows the various lengths of the constructs and their relative activities in the transient transfection system, standardized to the pGL3-Basic plasmid with no promoter.

In the HepG2 cells, the smallest fragment tested, 200 bp upstream of the PON1 gene, showed considerable promoter activity. Increasing the length of the promoter region had little effect except that a slight downward trend in expression level was observed with increasing lengths. The 5kb region produced only 35% luciferase activity compared to the 960 bp region (P = 0.04). The 11.5 kb region, however, produced somewhat more activity than the 5 kb fragment. Four of the promoters were tested for activity in the 293 cells, resulting in a similar pattern of expression (Fig. 1). The level of luciferase activity, however, was lower in the 293 kidney cells than the HepG2 liver cells.

A fragment almost 1 kb in length was chosen for sequence analysis. The published sequence from BAC GS1-155M11 was compared to the sequence we obtained from the YAC clone (A97E9), both generated from human genomic libraries. We identified five polymorphisms between the BAC and the YAC sequences at positions −108, −126, −162, −832 and −909, where the A of the ATG start codon is +1.
and the base immediately preceding it −1. We also sequenced the PON1 promoter regions from four individuals. We chose two individuals with very low PON1 activity levels and two with very high levels. We did not find any additional polymorphisms but the sequences did contain various combinations (haplotypes) of the polymorphisms at −108, −162, −832 and −909. The only instance of the −126C was in the BAC sequence (Table 1).

To analyse the functional consequences of the polymorphisms, the published BAC haplotype and three additional haplotypes were generated by site-directed mutagenesis and the various natural and generated haplotypes were subjected to transient transfection in HepG2 cells (Fig. 2). The haplotype found in the YAC clone produced a relatively low expression level while the G haplotype produced the highest expression level and the other haplotypes produced a range of intermediate expression levels. Comparison of the various haplotypes allowed determination of the effect of each polymorphism on expression level (Table 2). The polymorphisms at −126 and −832 had no statistically significant effect on expression level. The polymorphisms at −108, −162 and −909 each affected relative luciferase expression by approximately two-fold.

Discussion
In this study using cultured human cells, as observed previously in mouse (Primo-Parmo et al., 1996) and rabbit (Hassett et al., 1991) organs, we found that liver cells express more PON1 than kidney cells. The 293 kidney cells show the same pattern of expression levels as the HepG2 liver cells, but the degree of activation is lower relative to the background established by the promoterless plasmid. The data revealed that the promoter elements responsible for this cell-type-specific expression pattern are present in the first 200 bp upstream of the PON1 coding sequence. The CMV promoter driving the cotransfected renilla gene on the pRL-CMV control plasmid is strong and constitutively activated and has the potential to transactivate the test plasmid (Farr & Roman, 1992). The amount of control plasmid was kept low and the cell-type-specific expression pattern of the PON1 promoter fragments support the conclusion that

Table 2. Analysis of polymorphisms’ functional effects by comparison of haplotypes differing at only one position

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Haplotype</th>
<th>Expression Level</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>−108</td>
<td>TGGGC (YAC/A1)</td>
<td>1.0 ± 0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>CGGGC (E)</td>
<td>1.9 ± 1.0</td>
<td></td>
</tr>
<tr>
<td>−126</td>
<td>CCAAG (BAC)</td>
<td>3.0 ± 1.3</td>
<td>0.164</td>
</tr>
<tr>
<td></td>
<td>CGAAG (C1/C2)</td>
<td>2.1 ± 1.1</td>
<td></td>
</tr>
<tr>
<td>−162</td>
<td>CGGGG (A2/B1/D2)</td>
<td>1.2 ± 0.2</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>CGAGG (D1)</td>
<td>2.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGGAG (B2)</td>
<td>1.2 ± 0.5</td>
<td>0.050</td>
</tr>
<tr>
<td></td>
<td>CGAGG (C1/C2)</td>
<td>2.1 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGGGC (YAC/A1)</td>
<td>1.0 ± 0</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td></td>
<td>TGAGC (G)</td>
<td>3.6 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>−832</td>
<td>CGAAG (C1/C2)</td>
<td>2.1 ± 1.1</td>
<td>0.176</td>
</tr>
<tr>
<td></td>
<td>CGAGG (D1)</td>
<td>2.8 ± 1.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CGGGG (A2/B1/D2)</td>
<td>1.2 ± 0.2</td>
<td>0.991</td>
</tr>
<tr>
<td></td>
<td>CGGAG (B2)</td>
<td>1.2 ± 0.5</td>
<td></td>
</tr>
<tr>
<td>−909</td>
<td>CCAAG (BAC)</td>
<td>3.0 ± 1.3</td>
<td>0.040</td>
</tr>
<tr>
<td></td>
<td>CCAAC (F)</td>
<td>1.7 ± 0.6</td>
<td></td>
</tr>
</tbody>
</table>

*Polymorphism order, from left to right: −108, −126, −162, −832, −909; haplotype name in parentheses (see Table 1 for details; polymorphism that differs in italics). Expression level, fold induction relative to the YAC haplotype. t-test.
transactivation is not occurring, and that the firefly luciferase expression is a direct result of the PON1 promoter sequence.

As the length of PON1 promoter sequence increased, the reporter gene activity decreased, most notably with the 5 kb region which produced a luciferase activity level that was only 2.4-fold over background (compared to 9.5-fold for the 200 bp region). There may be a negative regulatory element(s) present that accounts for the reduction in activity. Counteracting sequences may be present elsewhere, such as in an intron. Since the 960 bp fragment produced good expression and contained the five polymorphisms, this fragment was chosen for further analysis.

Recently, Leviev and James (2000) published an analysis of polymorphisms they identified in the PON1 promoter (Genbank accession no. AF051133). They found three polymorphisms at −107, −824 and −907 that correspond to the polymorphisms described here at −108, −832 and −909, respectively. Additionally, Suehiro et al. (2000) identified polymorphisms at −108, −126 and −160, the last of which likely corresponds to the −162 polymorphism identified in this work. Base counting and sequence differences among the laboratories most likely account for the discrepancies. The sequences we obtained from the YAC clone and the individuals correspond to the sequence published for BAC GS1-155M11.

The polymorphisms were analysed for functional significance by comparison of haplotypes that differed at only one position, either using naturally occurring haplotypes isolated from the individuals or haplotypes generated by site-directed mutagenesis (Table 2). We did not detect a change in the reporter gene expression level for the −126 polymorphism. We did not find an effect by the −832 polymorphism either, although Leviev and James found that the −832 polymorphism produced a two-fold change in expression level (Leviev & James, 2000). Additionally, we detected an approximately two-fold change in expression level for the −909 polymorphism whereas Leviev and James found no effect. The discrepancies may be a consequence of interactions among the polymorphisms resulting in context effects. The exact haplotypes tested by Leviev and James were not listed in their publication, precluding comparison with the haplotypes described here.

The −T108C polymorphism resulted in a 1.9-fold change in expression level. The MatInspector program (Quandt et al., 1995), using the TRANSFAC database (Heinemeyer et al., 1999) identified a potential binding site for SP1, a ubiquitous activation factor, at position −108. The data described here are in agreement with Leviev & James (2000) and Suehiro et al. (2000).

The −162 polymorphism identified in this work resulted in a 1.8–3.6-fold change in expression level, depending on the haplotype context. The MatInspector program identified a potential NF-I transcription factor binding site when an A is present at −162, consistent with the observation that the higher expressing constructs are driven by −162A containing alleles. NF-I, also known as CTF, is a ubiquitous nuclear factor and a transcriptional activator (Nagata et al., 1982; Gronostajski et al., 1988; Santoro et al., 1988).

The data presented here support approximately two-fold effects for the −108, −162 and −909 polymorphisms individually. When the three polymorphisms are examined together, however, the effect does not appear to be additive. For example, the haplotype YAC/A1 has the lower activity version of all three polymorphisms while D1 has the higher activity versions. One would expect a six-fold difference in expression level yet the data showed only 2.8-fold activation. Additionally, the G haplotype has the highest expression level but only one of the three high activity polymorphisms (−162A). These results lend further support for context effects and interactions among the factors that bind in the vicinity of the polymorphisms.

Individual D is a heterozygote for the PON1 promoter region. One allele yields high expression in the transient transfection assay while the other yields low expression, yet the serum from this individual showed very high levels of PON1. These data support the hypothesis that PON1 DNA sequence may not be the sole determinant of PON1 expression levels. For example, cigarette smoke (Nishio & Watanabe, 1997) and smoking (James et al., 2000) have been shown to inhibit PON1. Thus, environment, diet (Shih et al., 1996; 1998) and interactions with other gene products (Mackness et al., 1987, 1989) are likely to have an effect on PON1 serum levels.

PON1 activity level variation among individuals is at least 13-fold (Furlong et al., 1989; Davies et al., 1996) and low levels of PON1 expression have been found to be associated with carotid artery disease (Jarvik et al., 2000). The presence of leucine at amino acid 55 is associated with higher mRNA and PON1 level on average (Blatter Garin et al., 1997; Leviev et al., 1997), although a given individual may have a PON1 expression level that is the opposite of that expected based on PON155 genotype (Brophy et al., 2000). As suggested previously (Leviev & James, 2000), the PON155 effect may be due to linkage to promoter polymorphisms rather than a direct effect by the coding region genotype. The previous reports
found that the PON1 promoter polymorphisms at $-107/-108$ and $-824/-832$ contribute to PON1 expression level. Here we have provided evidence that the $-162$ and $-907/-909$ polymorphisms also contribute to PON1 activity level and thus may contribute to an individual’s risk for heart disease and susceptibility to specific organophosphorus toxins. Interestingly, polymorphisms in the promoter of the HIV coreceptor gene CCR5 have been shown to affect disease progression in AIDS (Martin et al., 1998; Clegg et al., 2000), suggesting promoter polymorphisms may modulate the severity of many diseases.

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