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IgG-Paraoxonase-1 Fusion Protein for Targeted Drug Delivery Across the Human Blood-Brain Barrier

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Abstract

Paraoxonase (PON)-1 is the most potent human protein with organophosphatase activity against organophosphate (OP) toxins. OP compounds readily cross the blood-brain barrier (BBB), and have lethal mechanisms of action within the brain. The production of a brain penetrating form of human PON1, which crosses the BBB, is possible with the re-engineering of the enzyme as a fusion protein with a monoclonal antibody (MAb) against the human insulin receptor (HIR). The HIRMAb crosses the BBB via the endogenous insulin receptor, and acts as a molecular Trojan horse to ferry the PON1 into brain. The human PON1 was fused to the carboxyl terminus of the heavy chain of the chimeric HIRMAb. COS cells were dual transfected with the heavy chain gene and the light chain gene, and the HIRMAb-PON1 fusion protein was affinity purified with protein A chromatography. Western blotting with antibodies to human IgG or human PON1 showed the heavy chain of the HIRMAb-PON1 fusion protein was 40 kDa larger than the heavy chain of the chimeric HIRMAb. The ED₅₀ of binding to the HIR extracellular domain was 0.55 ± 0.07 nM and 1.1 ± 0.1 nM, respectively, for the chimeric HIRMAb and the HIRMAb-PON1 fusion protein. The PON1 enzyme activity of the fusion protein was approximately 25% of the enzyme activity in human plasma, based on a fluorometric enzymatic assay. In conclusion, human PON1 has been re-engineered as an IgG-organophosphatase fusion protein that penetrates the human BBB.

Keywords

blood-brain barrier; drug targeting; paraoxonase-1; chemical nerve gas

Introduction

Humans are subjected to organophosphate intoxication both chronically, in the form of pesticide exposure,¹ and acutely, in the form of chemical nerve gas agents.^{2,3} Serum esterases, such as butyrylcholinesterase (BCE) or paraoxonase (PON)-1, inactivate organophosphates (OP).⁴ PON1 organophosphatase activity is over 100-fold greater than BCE activity.⁵ Recombinant PON1 is a potential new treatment for OP intoxication.^{6–8} However, PON1 normally circulates bound to high density lipoprotein (HDL),⁹ and, in the absence of extracellular lipoprotein, PON1 is not secreted by cells.¹⁰ PON1 secretion by cells could be facilitated by the engineering and expression of IgG-PON1 fusion proteins, wherein the PON1 secretion is linked to the IgG secretion.

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New PON1 therapeutics for OP toxicity should be engineered to penetrate the central nervous system (CNS), following transport across the blood-brain barrier (BBB). Most OP molecules are low molecular weight, lipid soluble compounds that rapidly cross the BBB to enter the CNS. Moreover, the CNS is the principal site of action of the lethal effects of acute OP intoxication. The role of the CNS is implicated in several studies. Apnea and hypotension secondary to central muscarinic cholinergic stimulation by OPs occurs while diaphragm contractions can still be elicited by stimulation of the phrenic nerve.¹¹ Acetylcholinesterase (ACE) inhibitors, e.g., pyridostigmine, that are quaternary ammonium compounds, which do not cross the BBB, are less effective antidotes against OP toxicity as compared to tertiary amine ACE inhibitors, e.g., physostigmine, which do cross the BBB.¹² Muscarinic cholinergic receptor inhibitors, e.g., glycopyrrolate, that are quaternary ammonium compounds, which do not cross the BBB, are less effective antidotes against OP toxicity as compared to tertiary amine receptor blockers, e.g., atropine, which do cross the BBB.¹³

A large molecule such as PON1 does not cross the BBB. However, PON1 can be re-engineered to cross the BBB using molecular Trojan horse technology.¹⁴ A molecular Trojan horse is an endogenous peptide, or peptidomimetic monoclonal antibody (MAb), that crosses the BBB via endogenous receptor-mediated transport. A MAb against the human insulin receptor (HIR) has been genetically engineered and shown to rapidly cross the primate BBB in vivo.¹⁵ Similarly, genetically engineered fusion proteins of the HIRMAb also cross the BBB in vivo.^{16–18} Therefore, the purpose of the present investigation was to examine the feasibility of engineering, expressing, and validating a fusion protein of the chimeric HIRMAb and human PON1. The amino terminus of human PON1 is fused to the carboxyl terminus of the heavy chain of the HIRMAb, as shown in Figure 1. This configuration places the PON1 in a dimeric conformation, which replicates the native state of PON1, which forms a homo-dimer.¹⁹

Methods and Materials

Cloning of human PON1 cDNA

The human PON1 cDNA (GenBank accession # NM_000446) corresponding to amino acids Met¹-Leu³⁵⁵ was cloned by the polymerase chain reaction (PCR) using the oligodeoxynucleotides (ODNs) described in Table 1 and cDNA derived from reverse transcription of human liver PolyA⁺ RNA (Takeda/Clontech, cat# 6510–1). The forward (FWD) ODN primer introduces a Kozak sequence (i.e. CCCGACC) prior to the ATG initiation codon. The reverse ODN primer is complementary to the end of the open reading frame (orf) of PON1 plus 7 nucleotides of the 3'-untranslated region. The PON1 cDNA was cloned by PCR using 25 ng polyA⁺RNA-derived cDNA, 0.2 μ M forward and reverse ODN primers (Table 1), 0.2 mM deoxynucleosidetriphosphates, and 2.5 U PfuUltra DNA polymerase (Stratagene, San Diego, CA) in a 50 μ l Pfu buffer (Stratagene) containing 7% dimethylsulfoxide. The amplification was performed in a Mastercycler temperature cycler (Eppendorf, Hamburg, Germany) with an initial denaturing step of 95°C for 2 min followed by 30 cycles of denaturing at 95°C for 30 sec, annealing at 55°C for 30 sec and amplification at 72°C for 1 min; followed by a final incubation at 72°C for 10 min. PCR products were resolved in 0.8% agarose gel electrophoresis, and the expected major single band of ~1.1 kb corresponding to the human PON1 cDNA was produced (Figure 2A). The human PON1 cDNA was subcloned into the EcoRV site of the pcDNA3.1 eukaryotic expression plasmid, which was treated with alkaline phosphatase to prevent self ligation, and this PON1 expression plasmid is designated pCD-PON1. The engineering of this plasmid was validated by DNA sequencing in both directions, and by expression of PON1 in COS cells transfected with pCD-PON1.

Engineering of HIRMAb-PON1 expression vector

For the engineering of the pHIRMAb-PON1 heavy chain (HC) expression plasmid, the mature human PON1 cDNA corresponding to amino acids Met¹-Leu³⁵⁵ was cloned by PCR using the pCD-PON1 as template. The ODNs used for PCR are 5'-phosphorylated for direct insertion into the HpaI site of the pHIRMAb-HC expression plasmid (Figure 2B), as described previously.¹⁶ The pHIRMAb-HC plasmid encodes the HC of the chimeric HIRMAb, and dual transfection of COS cells with this plasmid and a light chain (LC) expression plasmid, pHIRMAb-LC, allows for transient expression of either the chimeric HIRMAb, or a fusion protein, in COS cells. The mature human PON1 forward PCR primer introduces "CA" nucleotides to maintain the open reading frame and to introduce a Ser-Ser linker between the carboxyl terminus of the CH3 region of the HIRMAb HC and the amino terminus of the PON1. The PON1 reverse PCR primer (Table 1) introduces a stop codon, "TGA," immediately after the terminal leucine of the mature PON1 protein. The engineered pHIRMAb-PON1 expression vector was validated by DNA sequencing.

The HIRMAb HC and LC cDNA expression cassettes are driven by the cytomegalovirus (CMV) promoter and contain the bovine growth hormone (BGH) polyadenylation (pA) sequence (Figure 2B). The engineering of the universal pHIRMAb-HC vector was performed by insertion of a single HpaI site at the end of the HIRMAb HC CH3 open reading frame (orf) by site directed mutagenesis (SDM), as described previously.¹⁶

Site-Directed Mutagenesis

The cloned human PON1 expressed Met-55 and Arg-192. The more active PON1 allozyme was produced with site-directed mutagenesis, as described previously.¹⁶ The SDM produced the following PON1 allozymes: Leu-55/Arg-192 and Leu-55/Gln-192. The SDM was verified by bi-directional DNA sequencing.

Transient expression of HIRMAb-PON1 fusion protein in COS cells

COS cells were dual transfected with pHIRMAb-LC and pHIRMAb-HC-PON1 using Lipofectamine 2000, with a ratio of 1:2.5, ug DNA:uL Lipofectamine 2000. Following transfection, the cells were cultured in serum free VP-SFM (Invitrogen, Carlsbad, CA), with or without 1% Ex-Cyte (Cellianca, Kankakee, IL), a lipoprotein supplement. The conditioned serum free medium was collected at 3 and 7 days. For screening assays, the COS cells were plated in 6-well cluster dishes. For production assays, the COS cells were plated in 10xT500 flasks with a proportionate scale-up of the Lipofectamine 2000 and the plasmid DNA.

Protein A affinity chromatography

Approximately 2 liters of serum free conditioned medium was collected over 7 days from 10xT500 flasks of COS cells dual lipofected with the pHIRMAb-HC-PON1 and pHIRMAb-LC plasmids. This medium was reduced to a 400 mL volume with tangential flow filtration, and the HIRMAb-PON1 fusion protein was purified by affinity chromatography with a 5 mL column of protein A Sepharose 4 Fast Flow (Amersham, Chicago, IL). The protein A column was equilibrated with buffer containing 1 mM CaCl₂, and the fusion protein was eluted with 0.1 M sodium acetate/pH=3.7/1 mM CaCl₂, followed by neutralization with 1 M Tris base. The neutralized acid eluate was concentrated and buffer exchanged with 0.01 M Tris/0.15 M NaCl/pH=7.4/1 mM CaCl₂ with an Ultra-15 concentrator (Millipore, Bedford, MA), and stored at -20°C. The protein content was measured with the bicinchoninic acid (BCA) assay (Pierce Chemical Co., Rockford, IL).

Human IgG ELISA

Human IgG ELISA was performed in Immulon-2 high binding plates (Dynex Tech., Chantilly, VA) with COS cell conditioned medium. A goat anti-human IgG primary antibody (Zymed-Invitrogen, Carlsbad, CA) was plated in 0.1 M NaHCO₃ (100 μ l, 2 μ g/ml) and incubated for overnight at 4°C. Plates were washed 0.01 M Na₂HPO₄/0.15 M NaCl/pH=7.4/0.05% Tween-20 (PBST), and blocked with 1% gelatin in PBST for 30 min at 22°C. Plates were incubated with 100 μ L/well of either human IgG1 standard or the fusion protein for 60 minutes at room temperature (RT). After washing with PBST, a goat anti-human kappa LC antibody conjugated to alkaline phosphatase (Sigma Chemical Co., St. Louis, MO) was plated for 60 min at 37°C. Color development was performed with p-nitrophenyl phosphate (Sigma) at pH=10.4 in the dark. The reaction was stopped with NaOH, and absorbance at 405 nm was measured in a BioRad ELISA plate reader.

Western blotting

The immunoreactivity of the HIRMAb-PON1 fusion protein was measured for both the human IgG part and the human PON1 part of the molecule. For human IgG Western blotting, the primary antibody was a goat anti-human IgG (H+L) antiserum from Vector Labs (Burlingame, CA), and binding was detected with a biotinylated horse anti-goat IgG (Vector Labs). For human PON1 Western blotting, the primary antibody was a mouse monoclonal antibody against human PON1 from Abcam (Cambridge, MA), and binding was detected with a biotinylated horse anti-mouse IgG (Vector Labs). Human IgG1 standard was from the Sigma Chemical Co. (St. Louis, MO), and the human PON1 standard was from American Research Products, Inc. (Belmont, MA).

HIR receptor assay

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with an ELISA using the lectin affinity purified HIR ECD. CHO cells permanently transfected with the HIR ECD were grown in serum free media (SFM), and the HIR ECD was purified with a wheat germ agglutinin affinity column, as previously described.²⁰ The HIR ECD (0.2 μ g/well) was plated on Immulon-2 high binding 96-well plates, and the binding of the chimeric HIRMAb, or the HIRMAb-PON1 fusion protein to the HIR ECD was detected with a biotinylated goat anti-human IgG (H+L) antibody (0.3 μ g/well), and the ABC Elite detection system (Vector Labs). The concentration that caused 50% binding to the HIR ECD, the ED₅₀, was determined by non-linear regression analysis using the WinNonlin software.

PON1 enzyme assay

PON1 enzyme activity was measured with a fluorometric assay using diethylphospho-6,8-difluoro-4-methyl-umbelliferone (DEPFMU) as the substrate,²¹ which was custom synthesized by Molecular Probes-Invitrogen (Carlsbad, CA). The assay buffer was 0.02 M Tris/pH=8.0/0.15 M NaCl/2 mM CaCl₂. The standard curve (3 to 300 pmol/tube) was generated with the reaction product, 6,8-difluoro-4-methylumbelliferone (Invitrogen). Human plasma was used as a positive control. Fluorometric readings were obtained with a Farrand filter flurometer using a filter with an emission wavelength of 460 nm and a filter with an excitation wavelength of 355 nm. Enzyme activity is reported as nmol/hour/mL. Enzyme activity was measured after both 20 and 40 min incubations, and was linear with respect to time of incubation.

Results

DNA sequencing of the expression cassette of the pCD-PON1 encompassed 2,196 nucleotides (nt), including a 714 nt CMV promoter, a 6 nt Kozak sequence, a 1,068 nt PON1 open reading

frame, and a 408 nt BGH sequence, which produced a 355 amino acid human PON1 protein, including the amino terminal Met, with 100% identity with the known sequence for human PON1 (NM_000446, AAB25717). The predicted molecular weight, minus glycosylation, of the PON1 was 39,773 Da with an isoelectric point (pI) of 5.15. The sequence analysis indicated the PCR cloned human PON1 was the Met-55/Arg-192 allozyme. SDM was used to convert the Met-55 residue to the more active Leu-55 (Methods).

Transfection of COS cells with pCD-PON1 resulted in an increase in PON1 enzyme activity in the medium, and the addition of 1% ExCyte lipid supplement resulted in a 19-fold increase in medium PON1 enzyme activity (Table 2). The medium PON1 enzyme activity was comparable to PON1 enzyme activity in 10% human plasma (Table 2).

The cDNA corresponding to the 355 amino acid PON1/Leu-55/Arg-192 was amplified by PCR using custom ODNs and the pCD-PON1 as template, and this cDNA was subcloned into the HpaI site of the pHIRMAb-HC plasmid, as outlined in Figure 2B. DNA sequencing of the expression cassette of the pHIRMAb-PON1 plasmid encompassed 3,560 nt, including a 714 nt CMV promoter, a 9 nt full Kozak site (GCCGCCACC), a 2,460 nt HIRMAb HC-PON1 fusion protein open reading frame, and a 303 nt BGH polyA sequence. The plasmid encoded for a 819 amino acid protein, comprised of a 19 amino acid IgG signal peptide, the 443 amino acid HIRMAb HC, a 2 amino acid linker (Ser-Ser), and the 355 amino acid human PON1. The predicted molecular weight of the heavy chain fusion protein, minus glycosylation, is 88,553 Da, with a predicted pI of 6.31.

Dual transfection of COS cells with the pHIRMAb-PON1 and the pHIRMAb-LC resulted in the secretion of detectable human IgG in the medium, as determined with a human Fc specific ELISA. The addition of 1% lipid supplement (ExCyte) to the medium did not result in an increase in secretion of the HIRMAb-PON1 fusion protein, based on the IgG ELISA. The HIRMAb-PON1 fusion protein was purified by protein A affinity chromatography from serum free medium conditioned by dual transfected COS cells. On Western blotting, the LC of either the HIRMAb or the HIRMAb-PON1 fusion protein react equally with a primary antibody directed against the human IgG (H+L), as shown in Figure 3A. The size of the HC of the fusion protein is about 40 kDa larger than the size of the HC of the HIRMAb on both Western blots using either the anti-human IgG primary antibody (Figure 3A) or the anti-human PON1 primary antibody (Figure 3B). The anti-PON1 primary antibody reacts with the HC of the fusion protein, and with recombinant PON1, but does not react with the HIRMAb (Figure 3B).

The affinity of the fusion protein for the HIR extracellular domain (ECD) was determined with a ligand binding assay using lectin affinity purified HIR ECD (Methods). There is comparable binding of either the chimeric HIRMAb or the HIRMAb-PON1 fusion protein for the HIR ECD with ED50 of 0.55 ± 0.07 nM and 1.1 ± 0.1 nM, respectively (Figure 4).

The PON1 enzyme activity of the protein A purified HIRMAb-PON1/Leu-55/Arg-192 fusion protein is shown in Table 3, in comparison with the PON1 enzyme activity in 20% human plasma. PON1 activity for the fusion protein is linear with respect to incubation time and concentration (Table 3). The PON1 enzyme activity of the HIRMAb-PON1/Leu-55/Arg-192 fusion protein, at a concentration of 68 μ g/mL, is comparable to the PON1 enzyme activity in 20% human plasma (Table 3). The PON1 enzyme activity against substrates such as paraoxon is higher for the Arg-192 allozyme, as compared to the Gln-192 allozyme, although enzyme activity against chemical nerve gas agents is higher for the Gln-192 allozyme.²² Therefore, SDM was used to convert the Arg-192 residue to the Gln-192 in the HIRMAb-PON1 fusion protein (Methods). Similar to paraoxon, the Arg-192 HIRMAb-PON1 allozyme has a higher enzyme activity against DEPFMU, the substrate used for the fluorometric assay (Table 4).

Discussion

The results of this study are consistent with the following conclusions. First, a bi-functional IgG-PON1 fusion protein has been genetically engineered, wherein human PON1 is fused to the carboxyl terminus of the heavy chain (HC) of a chimeric HIRMAb (Figure 1), and expressed and secreted in COS cells without lipid acceptor molecules (Results). Second, the HIRMAb-PON1 fusion protein is correctly processed in COS cells, resulting in the production of the expected size of the heavy chain of the fusion protein on IgG or PON1 Western blotting (Figure 3). Third, the HIRMAb-PON1 fusion protein is bi-functional and binds the HIR (Figure 4), and exhibits PON1 enzyme activity (Table 3). Fourth, the organophosphatase activity against DEPFMU of the Arg-192 fusion protein allozyme is higher, as compared to the Gln-192 fusion protein allozyme (Table 4).

The level of PON1 enzyme activity in the serum free medium conditioned by COS cells transfected with the pCD-PON1 plasmid was increased nearly 20-fold by the addition to the medium of 1% ExCyte, a lipoprotein supplement (Table 2). This observation parallels other reports that PON1 is secreted poorly by transfected eukaryotic cells, in the absence of a lipid acceptor.¹⁰ The finding that PON1 secretion is enhanced by the addition of a lipoprotein supplement to the medium (Table 2) suggests that binding of PON1 to lipoproteins is an obligatory requirement for PON1 secretion by cells. In contrast, the addition of ExCyte to the medium has no effect on the secretion of the HIRMAb-PON1 fusion protein (Results). Therefore, fusion of PON1 to an IgG molecule, which is normally secreted by cells, provides an alternative pathway for secretion of PON1 by transfected cells. Similarly, the HIRMAb-PON1 fusion protein is secreted to the medium without the requirement for lipid acceptors in the medium by permanently transfected Chinese hamster ovary (CHO) cells (unpublished observation).

Following transfection of COS cells, the HIRMAb-PON1 fusion protein heavy and light chains are correctly assembled, as demonstrated by the Western blotting of protein A affinity purified fusion protein (Figure 3). An anti-human IgG (H+L) primary antibody reacts with the light chain, and heavy chain, of both the chimeric HIRMAb and the HIRMAb-PON1 fusion protein, but not with PON1 (Figure 3A). Conversely, an anti-human PON1 primary antibody reacts with recombinant PON1, and the heavy chain of the HIRMAb-PON1 fusion protein, but not with the heavy chain of the chimeric HIRMAb (Figure 3B). There is comparable binding to the HIR extracellular domain of the chimeric HIRMAb and the HIRMAb-PON1 fusion protein (Figure 4). The high affinity of the HIRMAb-PON1 fusion protein for the HIR is attributed to the fusion of the PON1 at the carboxyl termini of the HIRMAb heavy chains (Figure 1). In contrast, the HIR binding sites on the HIRMAb-PON1 fusion protein are formed by the complementarity determining regions, which are located at the amino terminal portion of the HIRMAb-PON1 fusion protein.

The PON1 enzyme activity of the HIRMAb-PON1 fusion protein was measured in these studies with the fluorometric assay, which uses DEPFMU as the organophosphate substrate.²¹ PON1 enzyme activity is typically measured with paraoxon as the substrate using a spectrophotometric assay of human plasma samples.²³ However, given the limited production of the HIRMAb-PON1 fusion protein by transiently transfected COS cells, the more sensitive fluorometric assay was used in these studies. Human plasma was used as a positive control in the PON1 fluorometric assay. The PON1 enzyme activity of 20% human plasma was 63.5 ± 1.4 nmol/hr/mL (Table 3). Since the concentration of PON1 in human plasma is about 50 ug/mL,²⁴ the human plasma PON1 specific activity against the DEPFMU substrate is 6.3 nmol/hr/ug enzyme, as determined with the fluorometric assay. The PON1 specific activity against paraoxon in human plasma is 114 nmol/hr/ug enzyme, as determined with the spectrophotometric assay.²⁴ Therefore, the PON1 activity against DEPFMU is lower as

compared to paraoxon, and this observation parallels other reports showing a reduced catalytic rate constant for DEPFMU as compared to paraoxon for organophosphorus hydrolase.²¹ The PON1 enzyme activity of the HIRMAb-PON1/Leu-55/Arg-192 fusion protein is 165 ± 4 nmol/hr/ μ g (Table 3); therefore, the PON1 enzyme specific activity of the HIRMAb-PON1 fusion protein is 0.6 nmol/hr/ μ g protein. Since the molecular weight of the HIRMAb-PON1 fusion protein is about 3-fold greater than the PON1 homo-dimer, the PON1 enzyme specific activity of the HIRMAb-PON1 fusion protein is about 25% of the enzyme activity of the native PON1 in human plasma.

The PON1 enzyme exists as different allozymes with natural Met/Leu and Arg/Gln polymorphisms at amino acids 55 and 192, respectively.²² Enzyme activity is higher for the Leu-55 allozyme, as compared to the Met-55 allozyme,²⁵ and the HIRMAb-PON1 fusion protein described in these studies was engineered as the Leu-55 allozyme. With respect to position 192, HIRMAb-PON1 fusion proteins were engineered with either the Arg-192 or the Gln-192 polymorphism. The PON1/Arg-192 allozyme has a higher activity toward substrates such as paraoxon as compared to the PON1 /Gln-192 allozyme.²² Similarly, the HIRMAb-PON1/Arg-192 allozyme has a higher activity toward DEPFMU as compared to the HIRMAb-PON1/Gln-192 allozyme (Table 4). However, the Gln-192 allozyme may be the more potent therapeutic form of the fusion protein. The Gln-192 polymorphism confers on PON1 both a higher enzyme activity against chemical nerve gas agents,²² and a higher anti-atherogenic effect of the enzyme.²⁶

In summary, these studies describe a new IgG-PON1 fusion protein that is bi-functional, and both binds the HIR, to induce transport across the human BBB, and has PON1 organophosphatase enzyme activity (Figure 1). PON1 is the most potent human protein with organophosphatase activity.⁵ Re-engineering of human PON1 as a fusion protein with the HIRMAb not only allows for CNS targeting of the enzyme, but also the secretion of the enzyme by transfected host cells without lipid acceptors.

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Figure 1.

The HIRMAb-PON1 fusion protein is formed by fusion of the amino terminus of human PON1 to the carboxyl terminus of the CH3 region of the heavy chain of the chimeric HIRMAb. The fusion protein is a bi-functional molecule: the fusion protein binds the HIR, at the BBB, to mediate transport into the brain, and has PON1 organophosphatase enzyme activity.

VH=variable region of heavy chain; VL=variable region of light chain; CL=constant region of light chain; CH1, hinge, CH2, and CH3 are sub-domains of the constant region of the heavy chain of the HIRMAb.

Figure 2.

(A) Ethidium bromide stain of agarose gel of human PON1 cDNA (lane 1), which was produced by PCR from cDNA produced by reverse transcription of RNA from human liver, and PON1-specific ODN primers (Table 1). The expected band of ~1.1 kb band corresponding to the human PON1 cDNA is shown in lane 1. DNA standards are PhiX174 HaeIII digested DNA, 1.4, 1.0, 0.8, 0.6, 0.3-0.1 (lane 2); and Lambda HindIII digested DNA, 23, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.56 kb (lane 3). (B) Genetic engineering of pHIRMAb-PON1, the eukaryotic expression plasmid encoding the fusion protein of PON1 and the heavy chain (HC) of the chimeric HIRMAb. The fusion gene is 5'-flanked by the cytomegalovirus (CMV) promoter and 3'-flanked by the bovine growth hormone polyA (pA) sequence.

Figure 3.

Western blot with either anti-human IgG primary antibody (A) or an anti-human PON1 primary antiserum (B). The immunoreactivity of the HIRMAb-PON1 fusion protein (lane 2) is compared to the chimeric HIRMAb (lane 3) and to recombinant PON1 (lane 1). Both the HIRMAb-PON1 fusion protein and the HIRMAb have identical light chains on the anti-hIgG Western. The HIRMAb-PON1 fusion heavy chain reacts with both the anti-IgG and the anti-human PON1 antibody, whereas the HIRMAb heavy chain only reacts with the anti-IgG antibody. The size of the HIRMAb-PON1 fusion heavy chain, 95 kDa, is about 40 kDa larger than the size of the heavy chain of the HIRMAb, owing to the fusion of the 40 kDa PON1 to the 55 kDa HIRMAb heavy chain.

Figure 4.

Binding of either the chimeric HIRMAb or the HIRMAb-PON1 fusion protein to the HIR extracellular domain (ECD) is saturable. The ED_{50} of HIRMAb-PON1 binding to the HIR ECD is comparable to the ED_{50} of the binding of the chimeric HIRMAb.

Table 1

Oligodeoxynucleotide primers used in the RT-PCR cloning of human PON1

PON1 FWD: 5'-phosphate-CCGACCATGGCGAAGCTGATTG
PON1 REV: 5'-phosphate-CGGTCTGTTAGAGCTCACAGTAAAG

Table 2

PON enzyme activity in the medium of COS cells following transfection with pCD-PON1

Sample	PON1 enzyme activity (nmol/hr/mL)	
	0% ExCyte	1% ExCyte
COS medium, pCD-PON1	1.24 ± 0.01	23.3 ± 0.1
COS medium, Lipofectamine 2000	0.26 ± 0.02	0.29 ± 0.01
Human plasma (10%)	41.3 ± 3.1	n.m.

Mean ± SE (n=3 dishes per point); n.m.=not measured. Clone pCD-PON1 produces the Met-55/Arg-192 allozyme; serum free medium was harvested 7 days after transfection of COS cells with pCD-PON1.

Table 3

PON1 enzyme activity of protein A purified HIRMAb-PON1 fusion protein

Enzyme source	Enzyme assay units		PON1 enzyme activity (nmol/hr/mL or nmol/hr/ug)
	20 min	40 min	
Human plasma			
20%	140 ± 2	273 ± 1	63.5 ± 1.0
HIRMAb-PON1/Leu-55/Arg-192			
270 µg/mL	492 ± 6	999 ± 10	165 ± 4
68 µg/mL	117 ± 2	233 ± 1	156 ± 4

Fluorometric assay units were converted into enzyme activity based on the standard curve (Methods). The units of human plasma PON1 enzyme activity are nmol/hr/mL; the units of HIRMAb-PON1 enzyme activity are nmol/hr/ug protein.

Table 4

PON1 enzyme activity of protein A purified HIRMAb-PON1/Arg-192 and HIRMAb-PON1/Gln-192 fusion protein allozymes

Enzyme source	Assay units		
	10 min	20 min	40 min
HIRMAb-PON1/Leu-55/Arg-192			
270 µg/mL	184 ± 1	362 ± 2	717 ± 4
135 µg/mL	92 ± 1	183 ± 2	363 ± 2
68 µg/mL	45 ± 2	92 ± 1	181 ± 2
HIRMAb-PON1/Leu-55/Gln-192			
500 µg/mL	100 ± 2	200 ± 1	404 ± 2
250 µg/mL	52 ± 1	101 ± 2	203 ± 2
125 µg/mL	26 ± 2	52 ± 1	101 ± 2