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ORIGINAL ARTICLE

Adenovirus-mediated human paraoxonase1 gene transfer to provide protection against the toxicity of the organophosphorus pesticide toxicant diazoxon

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Human paraoxonase1 (hPON1) is a potential therapeutic against the toxicity of organophosphorus (OP) pesticides and chemical warfare nerve agents. We tested whether PON1 gene transfer using adenovirus provides protection against the toxicity of the OP diazoxon. Using an adenovirus construct containing hPON1 gene, we showed elevated levels of recombinant hPON1 *in vitro* in 293A cells and *in vivo* in mice. The recombinant enzyme was secreted by 293A cells into culture medium and into the systemic circulation of mice. Western blotting revealed that the virally expressed hPON1 had the expected molecular weight of 45 kDa. Recombinant hPON1 in mice was in complex with mouse high-density lipoprotein (HDL) and migrated more slowly than endogenous hPON1 in the human HDL complex. Mice injected with adenovirus expressed PON1 at 600–3480 U ml^{-1} on day 5 post-treatment, which is 8–50-fold above endogenous. Six mice expressing hPON1 survived $2LD_{50}$ doses of diazoxon. Four of the six mice survived a second dose of diazoxon (for a total of $4LD_{50}$) administered 24 h later. In contrast, none of the three mice in the control group survived one $2LD_{50}$ dose. These results show that hPON1 in mice functions as a prophylactic and offers significant protection against lethal doses of diazoxon.
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Keywords: gene delivery; adenovirus; paraoxonase1; organophosphorus pesticides; chemical warfare nerve agents

INTRODUCTION

Organophosphorus (OP) compounds, used worldwide as crop saving insecticides or as weapons of chemical warfare nerve agents, pose a substantial threat to soldiers and civilians alike irrespective of whether the nature of their misuse is hostile, terroristic, deliberate, accidental or intentional.[1,2] OP agents inactivate serine hydrolases acetylcholinesterase (AChE) and butyrylcholinesterase (BChE).[3] The acute toxicity[4] and the characteristic symptoms of OP poisoning are attributable primarily to inactivation of AChE, the enzyme responsible for the breakdown of the neurotransmitter acetylcholine. Sustained levels of acetylcholine at cholinergic synapses produce an acute cholinergic crisis characterized by miosis, increased tracheobronchial and salivary secretions, bronchoconstriction, bradycardia, fasciculation, behavioral incapacitation, muscular weakness and convulsions culminating in death by respiratory failure.[5] Current antidotal regimens for OP poisoning include anticholinergic drugs such as atropine sulfate to counteract the effects of elevated acetylcholine, oximes such as 2-PAM chloride to reactivate AChE and anticonvulsants such as diazepam to control OP-induced symptoms of tremors and convulsions.[6] These regimens are successful in preventing lethality due to OP poisoning, but are unable to prevent post-exposure incapacitation, convulsions, performance deficits or, in many cases, permanent brain damage.[7] These treatments have undesirable side effects and require administration within a prescribed time frame for maximal efficacy. The approved oximes have limited efficacy against chemical warfare nerve agent such as soman. Hence, a pre-treatment regimen, especially for military personnel in potential OP exposure scenarios would prove to be advantageous.

The short-acting carbamates, pyridostigmine and physostigmine, are the current choice of pre-treatment to bind targets on AChE preventing irreversible inactivation by OP. An alternate approach is the development of human proteins as bioscavengers that sequester OP before they reach and inhibit AChE.[8,9] Stoichiometric bioscavengers are the first generation prophylactics. Currently, plasma-derived human BChE[9–11] and Protexia, a recombinant BChE produced in the milk of transgenic goats, are under investigation.[12,13] BChE is a stoichiometric bioscavenger, requiring large quantities (200 mg per 70 kg person) of the enzyme to provide protection against $2LD_{50}$ of soman.[14] More recently, enzymes that can inactivate OP without being consumed are being investigated for their potential as catalytic bioscavengers.[15] An ideal candidate would be a stable, non-immunogenic or minimally immunogenic, non-toxic enzyme with a long half-life in the circulation that has high catalytic efficiency for OP hydrolysis.[16] Human paraoxonase1 (hPON1) has the potential to become such a candidate. In particular, the PON1 Q192 isoform is most promising, as it was found to hydrolyze nerve agents sarin and soman more rapidly than the PON1 R192 allele.[17]

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The hPON1 (EC 3.1.8.1; formerly EC 3.1.1.2) is a calcium-dependent glycoprotein, 354 amino acids in length with an estimated molecular mass of 45 kDa.[18,19] Carbohydrate structures account for 15% of its molecular mass.[19,20] A number of studies have provided evidence that PON1 decreases the toxicity of certain OP agents *in vivo*.[21,22] PON1 knockout mice were found to be more sensitive to chlorpyrifos and diazinon[23] than control mice, suggesting the ability of PON1 to metabolize these toxic compounds *in vivo*. In addition, adenoviral vectors containing the gene for PON1 increased serum PON1 activity about 60% and protected mice against the toxicity of chlorpyrifos.[24] However, the usefulness of PON1 in alleviating the toxicity of a nerve agent, such as soman, sarin or VX, remains to be examined.

To overcome the technical challenges associated with testing native or recombinant enzymes for *in vivo* efficacy studies, gene delivery using viral vectors such as adenovirus is an attractive approach that has been adopted widely.[25] Using high titer adenovirus-expressing human PON1Q192 alloform (hereafter referred to as hPON1), we show persistent and elevated levels of recombinant hPON1 *in vitro* in HEK-293A cells and *in vivo* in mice. Mice that have 8–10 times the endogenous level of PON1 activity are protected from the lethality of diazoxon.

RESULTS

Expression and dose response of adenovirus-expressing hPON1 in HEK-293A cells

The ability of adenovirus-expressing hPON1 (Ad-hPON1) to express recombinant hPON1 was evaluated in HEK-293A cells. Media from cells infected with various levels of Ad-hPON1 (0–50 plaque-forming units (pfu) per cell) were assayed for PON1. As shown in Figures 1a (phenylacetate substrate) and 1b (paraoxon substrate), PON1 was detected in the culture media and increased both with incremental levels of virus and time post-infection. Maximal enzyme activity was obtained using 25–50 pfu per cell on 4–5 days post-infection. No further increase in activity was obtained by increasing the virus beyond 50 pfu per cell (data not shown). A quantitative determination of recombinant hPON1 activity in the cell culture medium and cytosolic fractions on day 5 post-virus infection revealed that 95% of the PON1 activity was in the medium and only 5% was within the cytosol (data not shown).

PON1 expressed by 293A cells was partially purified using ammonium sulfate fractionation and metal-affinity chromatography. Partially purified hPON1 was detected on a western blot with anti-6×histidine antibody. The results shown in Figure 2 suggest that the recombinant hPON1 has a molecular weight ~45 kDa and thus appears to be full length and intact. These results suggest that hPON1 was synthesized and secreted by HEK-293A cells in full-length form with the appropriate number of carbohydrate structures.

Recombinant hPON1 protein expressed in mouse plasma

The Ad-hPON1 was introduced into mice by a single injection via the tail vein. The expression of active recombinant hPON1 enzyme was determined in plasma over a period of 10 days. The activity profiles of PON1 using phenylacetate and paraoxon as substrates are depicted in Figure 3. Each time point in the figure represents mean ± s.e. of values from 6 to 8 mice. The plasma enzyme activities begin to rise rapidly by day 1, reaching peak levels on days 3 to 5 and then declining back to baseline by day 10. The PON1 activity in one mouse increased 50-fold above baseline levels on day 3. This mouse was included in Figure 3. Overall, 8–50-fold increases in PON1 activity levels (613–3480 U ml^{-1}) were achieved over the baseline PON activity (70–120 U ml^{-1}) on days 3 to 5 following Ad-hPON1 injection. Importantly, mice tolerated increased levels of hPON1 over extended periods with no overt symptoms of toxicity observed. These results show that a single injection of Ad-hPON1 was capable of introducing high levels of enzymatically active recombinant hPON1 into systemic circulation of mice and that these levels of hPON1 appear to be non-toxic.

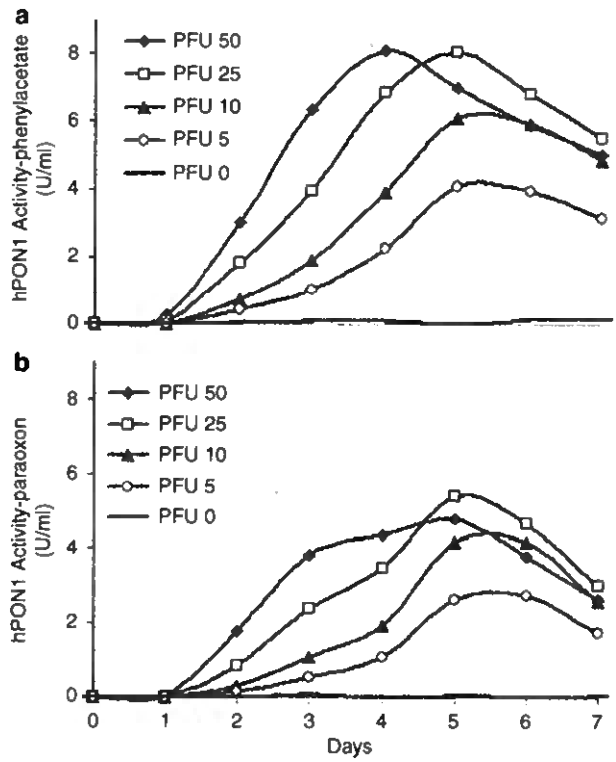

Figure 1 Recombinant hPON1expression in HEK-293A cells as measured by PON1 activity using phenylacetate (**a**) and paraoxon (**b**) as substrates. The HEK-293A cells were plated in six-well dishes 1×10^6 cells per well and grown for 24 h before infection with Ad-hPON1 at the indicated pfu. Medium from cultures grown for the indicated number of days was assayed for PON1 activity. The results depicted are representative of several independent experiments using different batches of viral preparations and/or sources.

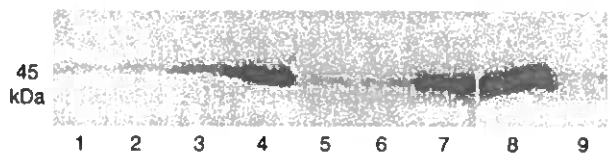

Figure 2 Sodium dodecyl sulfate (SDS)–PAGE of hPON1 expressed and secreted by HEK-293A cells. The Cobalt-affinity resin column fractions were subjected to sodium dodecyl sulfate (SDS)–PAGE and western blotting with an antibody to 6× His to determine the molecular size of recombinant hPON1 expressed by Ad-hPON1 in HEK-293A cells. The lanes are 1, sample before loading onto the column; 2, flow through; 3–4, wash fractions with equilibration buffer and 5–9, elution fraction with 500 mM imidazole. A majority of recombinant hPON1 was found in the elution fractions 3 and 4 (lanes 7 and 8) and its molecular size was estimated to be 45 kDa.

Recombinant hPON1 protein expression in mouse tissues

On day 4 post-injection, at the peak of PON1 plasma activity, two Ad-hPON1 mice and one Ad-control mouse were euthanized for

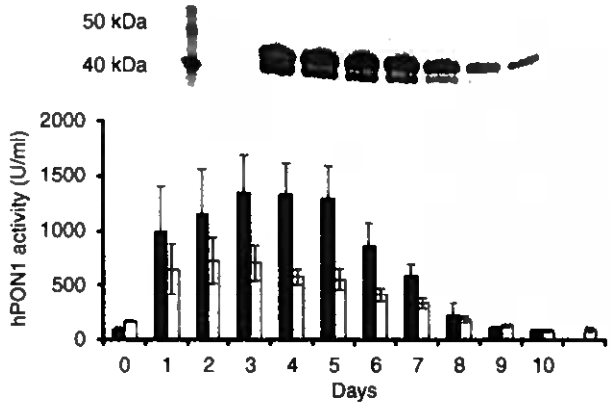

Figure 3 PON1enzyme activity on various days in plasma of mice injected with Ad-hPON1 (n=8). Blood samples from mice were collected on days 0 to 11 before and after Ad-hPON1 injection. Plasma samples were analyzed for PON1 activity with the substrates phenylacetate (black bars) and paraoxon (white bars) as described in Materials and methods. One unit of PON1 activity is defined as 1 μmol phenylacetate or 1 nmol paraoxon hydrolyzed per min under standard assay conditions. Activities plotted were mean ± s.e. Inset: Expression and secretion of hPON1 in mouse serum as a function of time. Plasma samples from a mouse that showed maximal expression of hPON1 were analyzed by sodium dodecyl sulfate (SDS)–PAGE followed by western blotting using anti-hPON1 polyclonal antibodies.

Table 1 Distribution of human PON1 activity determined using phenylacetate as the substrate in tissues and plasma on day 4 following Ad-hPON1 IV injection into two mice and one control mouse

	Ad-hPON1 mouse ($U\,mg^{-1}$)	*Ad-hPON1 mouse* ($U\,mg^{-1}$)	*Control mouse* ($U\,mg^{-1}$)
Plasma ($U\,ml^{-1}$)	613	1370	94
Liver	191	202	157
Brain	3	4	3
Salivary glands	12	7	7
Lung	16	15	18
Heart	5	6	2
Diaphragm	3	4	3
Stomach	13	13	16
Intestine	202	188	202
Fat	6	7	6
Muscle	4	4	3
Testes	6	5	3
Spleen	5	4	6
Kidney	51	53	50
Skin	5	5	4
Bladder	3	5	4

Abbreviations: Ad-hPON1, adenovirus-expressing hPON1; hPON1, human paraoxonase1.
Control mice received the Ad-control virus in the same manner as Ad-hPON1 mice.

determination of PON1 tissue activity. Activity was determined using phenylacetate as the substrate. The two Ad-hPON1-injected mice had plasma levels 7- and 15-fold higher than those in the control mouse (Table 1). In contrast, activity levels of PON1 in all the tissues examined of Ad-hPON1-injected mice were similar to those in control virus-injected mouse. These results suggest that the recombinant hPON1 transduced through adenovirus was found primarily in the systemic circulation.

Size of hPON1 expressed in mouse plasma

To determine if the virus-expressed recombinant hPON1 was full length and intact, plasma from the mouse expressing recombinant hPON1 at 3482 $U\,ml^{-1}$ on day 3 was analyzed by western blotting with rabbit polyclonal anti-hPON1 peptide antibodies. The antibody recognized a doublet of ~45 and ~42 kDa in all the samples analyzed (days 1 through 7) (Figure 3 inset). The lower migrating form likely represents incompletely glycosylated hPON1 as such a phenomenon is well known at very high expression levels of glycoproteins.[26,27] Plasma from pre-injection bleed (day 0) did not contain detectable amounts of hPON1 protein. The antibody does not appear to cross-react with mouse PON1. Also notable is that despite the very high levels introduced into circulation, no proteolysis was observed. The results in Figure 3 also show correlation of PON1 activity with PON1 protein bands. The activity levels are in excellent agreement with the density of bands on the gel. These results show that recombinant hPON1 expressed in mice is full length and intact and that it remains in an intact form in the circulation as long as 7 days.

Analysis of PON1enzyme activity in mouse plasma by non-denaturing polyacrylamide gel electrophoresis

As native PON1 associates with high-density lipoprotein (HDL) *in vivo*, we analyzed recombinant hPON1 in mouse plasma by non-denaturing polyacrylamide gel electrophoresis (PAGE) to identify if the overexpressed hPON1 is capable of associating with mouse HDL. We chose mouse plasma containing a high level of recombinant hPON1 (3482 $U\,ml^{-1}$ on day 3 post-virus injection). The esterase activity in gels was identified by staining with β-naphthylacetate and Fast blue RR (Figure 4a). The β-naphthylacetate detects not only PON1, but also BChE, carboxylesterase (CES) and albumin (ALB) esterase. BChE is the weak band near the top of lane 1. CES is the intense band that migrates just above ALB in lanes 2 and 3. ALB is the weak band in lane 1. PON1 is the broad, heavily stained band in lane 3, and the broad fuzzy band in lanes 1 and 2. Positive identification of the PON1 band was obtained by preincubating the gel with 10 mM EDTA, a specific inhibitor of PON1. Figure 4a, lane 6, shows that the hPON1 band nearly disappears in the presence of EDTA, while all other bands remain. The hPON1 in human plasma (Figure 4a lane 4) and mouse PON1 in control mouse plasma (Figure 4a, lane 5) also disappear in the presence of EDTA. The band for PON1 was identified with a second set of inhibitors, diisopropylfluorophosphate (DFP) and diazoxon, specific inhibitors of the serine esterases, BChE and CES. These OP do not inhibit PON1. Figure 4a, lanes 9 and 12, shows that activity of hPON1 is unaffected by 0.2 mM DFP and 0.2 mM diazoxon that inhibit the activity of BChE and CES. Similarly, Figure 4a, lanes 8 and 11, shows that endogenous mouse PON1 is resistant to inhibition by DFP and diazoxon, whereas bands for BChE and CES disappear. The band for human BChE in Figure 4a, lanes 7 and 10, disappears in the presence of DFP and diazoxon. Human plasma contains no CES,[28] as confirmed by the absence of a band for CES in Figure 4a lane 1. ALB stains weakly for esterase activity under all conditions. The location of the ALB band was confirmed by the intense Coomassie blue-stained band in all lanes of Figure 4b. Mouse ALB (Figure 4b lanes 2 and 3) migrates more rapidly than human ALB (lane 1).

Wild-type mouse plasma has a band of PON1 activity that migrates at a position similar to that of recombinant hPON1 expressed in mouse plasma (Figure 4a, lanes 2 and 3). It appears that all of the overexpressed hPON1 associates with mouse HDL. Endogenous hPON1 in human plasma (lane 1) migrates more rapidly than hPON1 in mouse plasma (lane 3) and endogenous mouse PON1 in mouse plasma (lane 2). This suggests that hPON1 in the human HDL

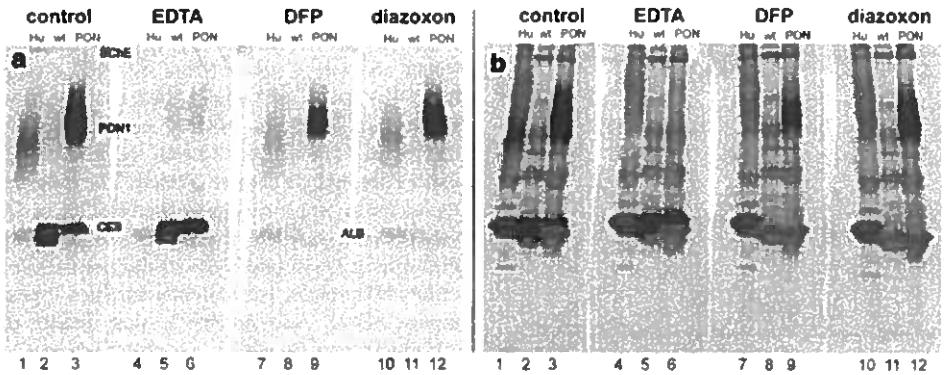

Figure 4 Analysis of plasma PON1 by activity staining in non-denaturing PAGE gels. (**a**) Plasma samples and gel sections were preincubated with inhibitors (10 mM EDTA, lanes 4–6; 0.2 mM DFP, lanes 7–9 or 0.2 mM diazoxon, lanes 10–12) or without any inhibitor (control, lanes 1–3) for 30 min at room temperature before the gels were stained for esterase activity with β-naphthylacetate and Fast Blue RR. Lanes 1, 4, 7, 10 contain 1 μl human serum. Lanes 2, 5, 8, 11 contain 1 μl wild-type mouse serum (70 U ml^{-1} PON1 activity). Lanes 3, 6, 9, 12 contain 1 μl mouse plasma expressing 3482 U ml^{-1} of hPON1. A band for the butyrylcholinesterase (BChE) tetramer is near the top of the gel. PON1 makes a complex with high-density lipoprotein and migrates as a broad band. Carboxylesterase (CES) migrates just above albumin (ALB) in mouse plasma. Human plasma contains no carboxylesterase. (**b**) Gels were counter-stained with Coomassie blue to confirm the location of the albumin band.

complex carries more negative charges than hPON1 in the mouse HDL complex.

Recombinant hPON1 associates with mouse HDL *in vivo*

To identify if Ad-hPON1-transduced recombinant hPON1 was capable of associating with mouse HDL, we performed non-denaturing PAGE analysis with purified hPON1, control mouse plasma and plasma from three Ad-hPON1-treated mice. Each of the plasma samples contained different hPON1 activity levels (942, 1656 and 2568 U ml^{-1}). PON1 activity was identified by staining with β-napthyl acetate and Fast blue RR, while the presence of HDL in the samples was detected by using Sudan Black B. The results are illustrated in Figure 5.

The mouse plasma control shows a light band of diffuse PON1 activity with a diffuse band of HDL at the same gel location (lanes 1a and b). The purified hPON1 shows a diffuse band of PON1 activity with no corresponding HDL because the lipoprotein was removed during the purification process (lanes 2a and b). Plasma from the Ad-hPON1-treated mice showed broad diffuse bands of PON1 activity with intensities that correlate to the known activity levels (Figure 5, PON activity, lanes 4a, 5a, and 6a). Broad diffuse bands of HDL are observed in positions in the Sudan Black B-stained gel (Figure 5, Sudan Black, lanes 4b, 5b, and 6b) that correspond to the PON activity stained section. Similar results were obtained when these samples were processed on agarose gels (data not shown). These results show that the virus-transduced recombinant hPON1 associates with HDL in mouse plasma.

Protection of mice from OP toxicity by Ad-hPON1

We addressed the question of whether recombinant hPON1 expressed in mice at high levels would protect the animals from OP toxicity. As maximal PON1 levels were observed on days 3–5 post-treatment with Ad-hPON1, mice were challenged with diazoxon on day 4. Mice surviving the first dose of 28 mg kg^{-1} ($2LD_{50}$ dose) for 24 h were challenged again with another $2LD_{50}$ dose of diazoxon and those that survived the second $2LD_{50}$ dose for a minimum of 24 h received a third dose. Control mice (n=3) and the treatment group (n=6) were monitored for cholinergic symptoms following each dosing regimen. Surviving mice had cholinergic signs of toxicity only briefly. All signs

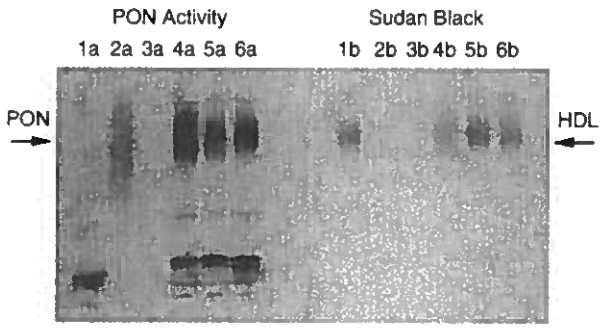

Figure 5 PON1 activity and HDL in the plasma of control and adenovirus-treated mice and in purified human PON1. Lanes 1a–6a were stained for PON1 activity with β-napthyl acetate. Lanes 1b–6b were stained for lipoproteins (HDL) with Sudan Black B. Lanes 1a and b contain control mouse plasma (1 μl) (70 U ml^{-1}). Lanes 2a and b contain purified hPON1 (12.5 μl) (21 U ml^{-1}). Lanes 3a and b are blank. Plasma (1 μl) from Ad-hPON1-treated mice are in lanes 4a and b (2568 U ml^{-1}), 5a and b (942 U ml^{-1}) and 6a and b (1656 U ml^{-1}). Arrows point to the location recombinant hPON1–HDL complexes in both gels.

of toxicity had disappeared by the time they were rechallenged with another dose of diazoxon. Observations of toxicity and time to death or morbidity are presented in Table 2.

The Ad-hPON1-injected mice, on average, had sevenfold higher levels of PON1 activity on day 3 compared with day 0 (pre-Ad-hPON1). All three mice in the control group were moribund within 2 h of receiving the $2LD_{50}$ dose. In contrast, all six mice of the treatment group survived the first $2LD_{50}$ for 24 h. Four of the Ad-hPON1 mice survived a second $2LD_{50}$ dose for 24 h and received a third $2LD_{50}$ dose. The four animals receiving a total of $6LD_{50}$ were moribund on average 20 min after receiving the last dose. PON1 levels in the two mice that were moribund after $4LD_{50}$ were relatively lower (378 ± 67 U ml^{-1}) than the levels in the four mice that received the third $2LD_{50}$ dose (576 ± 116 U ml^{-1}). Overall, these results suggest that high levels of recombinant hPON1 in mice function as prophylactics that offer excellent protection against lethal doses of diazoxon. The protection level correlates with plasma PON1 levels.

Table 2 Observations of cholinergic signs and toxicity following diazoxon challenge of control mice and of mice expressing hPON1

	hPON1-treated mice (n=6 males)			*Control mice (n=3 males)*
Mean body weight (±s.d.)		22.2±0.7 g		22.0±1.7 g
Mean hPON1 activity pre-treatment (±s.d.)[a]		73±6.8 U ml^{-1}		75.2±6.6 U ml^{-1}
Mean hPON1 activity day 4 post-virus (no diazoxon) (±s.d.)[a]		524±134 U ml^{-1}		78.3±2.2 U ml^{-1}
Cumulative dose	$2LD_{50}$ (day 4 post-virus)	$4LD_{50}$ (day 5 post-virus)	$6LD_{50}$ (day 6 post-virus)	$2LD_{50}$ (day 4 post-virus)
Cholinergic signs	*No. of animals with symptom post-challenge/total no. challenged*			
Reduced motor activity	(6/6)	(6/6)	(4/4)	(3/3)
Muscle fasciculation	(3/6)	(3/6)	(4/4)	(3/3)
Whole body tremors	(0/6)	(2/6)	(4/4)	(3/3)
Hunched posture (dormouse)	(3/6)	(3/6)	(4/4)	(3/3)
Lacrimation (mucous)	(1/6)	(5/6)	(4/4)	(3/3)
Reduced handling reaction	(5/6)	(5/6)	(4/4)	(3/3)
Splayed hind legs	(0/6)	(4/6)	(2/4)	(3/3)
Ataxic gait	(0/6)	(4/6)	(2/4)	(3/3)
Salivation	(0/6)	(4/6)	(2/4)	(3/3)
Piloerection	(3/6)	(6/6)	(4/4)	(3/3)
Mean body temperature (°C) 120 min post-challenge or before death	38.1±0.7	33.8±2.4	30.8±1.8	30.2±0.5
Moribund/died	0/6	2/6	4/4	3/3
Mean time post-challenge (min)	NA	120	30	75

Abbreviations: Ad-hPON1, adenovirus-expressing hPON1; hPON1, human paraoxonase1.
[a]hPON1 activity was determined using phenylacetate as the substrate. Control mice received the Ad-control virus in the same manner as Ad-hPON1 mice.

Observations made following the diazoxon challenge indicated that protection was afforded to the Ad-hPON1 treatment group. After the first $2LD_{50}$ dose, the Ad-hPON1 group had no whole body tremors, reduced lacrimation, no splayed hind legs, no ataxic gait, no salivation and reduced piloerection (Table 2). Decrease in body temperature serves as a quantitative measure of toxicity. The average body temperature of the control group 2 h post-challenge or immediately before euthanasia was 30.2±0.5 °C compared with 38.1±0.7 °C measured at the same time in the Ad-hPON1-treated mice.

DISCUSSION

The hydrolytic activity of PON1 against certain OPs renders it an attractive therapeutic candidate for prevention and/or the treatment of toxicity from exposure to OP pesticides and nerve agents. As PON1 is a glycoprotein and optimally functional in complex with HDL, there are technical challenges and constraints in generating large quantities of robust PON1 for therapeutic evaluation and use. The results presented in this manuscript suggest that using adenoviral vectors to introduce PON1 into systemic circulation is a viable approach. Methods for delivering PON1 for protection against OP toxicity are summarized below.

Adenovirus

Our results show that (1) recombinant hPON1 clearly functions in prophylactic manner against OP toxicity when the levels in blood circulation are high and (2) PON1 has therapeutic potential as we were able to continually escalate the dose to $6LD_{50}$ over a 3-day period. The PON1 activity levels in our studies were estimated to be up to 50-fold higher than the pre-adenovirus levels in some mice. A previous study using adenovirus to introduce hPON1 into mice reported a 60% increase in serum PON1 activity and prevention of toxicity[24] from chlorpyrifos as determined by inactivation of brain AChE levels. Studies addressing atherosclerosis through Ad-PON1 gene transfer were more successful, achieving 4–15-fold higher levels of PON1 in mice and improved vascular wall oxidative stress, endothelial cell function, smooth muscle cell Ca^{2+} homeostasis and reduced oxidized LDL.[29,30]

Purified rabbit and hPON1

Protection by PON1 from OP toxicity has been achieved by injecting purified rabbit PON1 into rats and mice. By increasing the PON activity 30- to 50-fold above endogenous levels, rats and mice were protected from the toxicity of chlorpyrifos oxon as measured by inhibition of AChE activity.[21,31] Treatment with purified rabbit PON1 30 min after poisoning mice with the phosphorothioate chlorpyrifos prevented all cholinergic signs of intoxication.[22]

Engineered recombinant hPON1 containing the mutation K192 was expressed in *Escherichia coli* and purified. Post-treatment of PON1 knockout mice with the purified recombinant hPON1 protected the mice from the lethal effects of diazoxon.[16] A chimeric PON1 85% identical in amino-acid sequence to hPON1 was expressed in *E. coli* and used for protection experiments in mice.[32] It was found that the chimeric PON1 protected from the lethality of chlorpyrifos oxon.

Naked DNA

Naked DNA injected into mice as a plasmid encoding hPON1 decreased the mortality from soman exposure.[33]

Complex of recombinant hPON1 with mouse HDL

Our results show that the hPON1 expressed by adenovirus in mice spontaneously associated with mouse HDL. These results have clear implications for analyzing the meaningful biological function(s) of any PON1 therapeutic testing, as such a complex formation is well known to be a pre-requisite for maintaining stable PON1 activity in serum.[34,35] In addition, these results show that adenoviral gene transfer results in functional PON1–HDL complexes, without resorting to extraordinary genetic engineering means to generate such complexes.[32] A study which compared the effectiveness of pre-treatment with recombinant chimeric PON1 to pre-treatment with a PON1–HDL complex found that the recombinant chimeric PON1

on its own was more effective at protecting mice from chlorpyrifos oxon toxicity. It was hypothesized that upon intravenous administration, the recombinant chimeric PON1 associates with endogenous HDL particles that stabilize it and slow its clearance rate.[32] In this regard, while purified hPON1 was shown to associate with HDL when added to whole serum,[36] the fate of bacterially produced hPON1 injected into mice was not studied.[16] Our results not only provide direct evidence for such association between recombinant hPON1 and mouse HDL, but also show functional activity of such complexes.

Summary

We previously reported that gene transfer using adenovirus can be a promising strategy and can be effective for evaluating potential biological agents against chemical weapons *in vivo*.[37,38] More recently, we found that these biological agents, namely truncated and full-length human BChE and mouse BChE, protected mice from multiple LD_{50} doses of the most toxic nerve agent VX and the pesticide echothiophate (Chilukuri *et al.*, unpublished data). This study extends these results to PON1Q192 alloform, considered to be the most promising for pre-treatment in the category of catalytic bioscavengers against OP poisoning. We show that high levels of hPON1 delivered to mice through adenovirus means are highly effective in preventing OP toxicity. Excellent protection against lethal doses of diazoxon was notable as four of six mice survived $4LD_{50}$ doses administered as two $2LD_{50}$ doses 24 h apart. Moreover, the results show protection afforded to the treatment group in terms of a reduction in cholinergic signs of toxicity. Based on these results, we advocate continued investigations into gene transfer for thorough and systematic evaluation of PON1 as a prophylactic and therapeutic agent for possible military as well as civilian use. Current first generation adenoviral vectors may not be ideal for human use due to immunogenic or safety considerations. A safer gene delivery system approved for human use would deliver therapeutic levels of PON1 against OP and nerve agent poisoning.

MATERIALS AND METHODS

Reagents and cells

AdenoVATOR vector system cloning kit and HEK-293A human embryonic kidney epithelial cells were obtained from MP Biomedicals (Irvine, CA, USA). Pfu DNA polymerase, calf intestinal alkaline phosphatase and DNA ligase were purchased from Promega Corp. (Madison, WI, USA). Restriction endonucleases, BglII, Pme I and Pac I, were purchased from New England Biolabs (Beverly, MA, USA). DNA purification kits were obtained from Qiagen Inc. (Valencia, CA, USA). Lipofectamine 2000, cell culture media, medium supplements and trypsin-EDTA were purchased from Life Technologies Inc. (Gaithersburg, MD, USA). Monoclonal antibody to 6×histidine tag was purchased from AbCam (Cambridge, MA, USA). PVDF membrane and the ECL plus western blotting detection kit were purchased from GE Healthcare Sciences (Piscataway, NJ, USA). Rabbit polyclonal antibodies to hPON1 (Cat#P0123), DFP, β-naphthylacetate, Fast blue RR and all other chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA) or Quality Biological Inc. (Gaithersburg, MD, USA). The source for diazoxon was Chem Service Inc. (West Chester, PA, USA).

Production of recombinant adenovirus-expressing hPON1

Recombinant Ad-hPON1 (gi:209413720 in the NCBI database) was generated as described in the applications manual (MP Biomedicals). Briefly, hPON1 cDNA (Q192 alloform) containing a 6×histidine tag at its carboxyl terminus was used in a polymerase chain reaction to introduce BglII restriction endonuclease sites at 5′ and 3′ ends of the gene. The primers used in the PCR reaction were 5′-GAAGATCTATGGCGAAGCTGATTG-3′ (forward) and 5′-GAAGATCTTTACTAATGATGGTGATG-3′ (reverse). The PCR product was cloned into adenoviral transfer vector, pAdenoVator-CMV5-IRES-GFP, linearized previously with restriction endonuclease BglII. The insert was fully sequenced to ensure that its nucleotide sequence was intact. The left and right arm of the pAdenoVator-CMV5-IRES-GFP vector contains Ad5 nucleotide sequences necessary to mediate homologous recombination with the Ad5dE1/dE3 vector in *E. coli* plus inverted terminal repeat and packaging signal sequences required for viral production in mammalian cells. For generation of recombinant adenovirus plasmids, pAdenoVator-CMV5-IRES-GFP-hPON1 plasmid was linearized with PmeI and co-transformed with Ad5dE1/dE3 backbone plasmid into recombination proficient BJ5183 cells. Recombination was confirmed by PacI digestion and one of the recombined clones (pAd-hPON1) was retransformed into DH5α cells for large-scale plasmid preparation.

For the production of recombinant Ad-hPON1 HEK-293A cells, which produce the deleted E1 genes in *trans* were transfected with 4 μg of Pac-1-digested pAd-hPON1 plasmid and Lipofectamine 2000. Cells were harvested 9 days post-transfection and re-suspended in Hanks Balanced Salt Solution and crude recombinant virus was collected after four freeze-thaw cycles. The low titer crude virus was sent to MP Biomedicals or Welgen Inc., Worcester, MA, USA for single clone selection, amplification, purification, viral titers and quality testing. Most *in vitro* assays were carried out with the Qbiogene/MP Biomedicals preparations and the *in vivo* experiments used the virus supplied by Welgen. The control adenovirus lacked the hPON1 cDNA, but was otherwise identical to the hPON1-expressing adenovirus.

Cell culture and infection with Ad-hPON1

HEK-293A cells (1×10^6) were seeded in six-well plates. After 24 h, the cells were infected with 0–50 pfu of Ad-hPON1 for 1–2 h using 500 μl of infection medium (Dulbecco's modied Eagle's medium containing 2% FBS, antibiotics penicillin and streptomycin, and sodium pyruvate) at 37 °C. Following infection, 2.5 ml of growth medium (Dulbecco's modied Eagle's medium containing 10% FBS, 50 μg ml^{-1} penicillin and streptomycin, L-glutamine and 50 μg ml^{-1} sodium pyruvate) was added to the cells and incubated at 37 °C. The PON1 activity in the culture medium was quantified using paraoxon and phenyl acetate as the substrates.[19]

In one experiment, hPON1 activity was quantitatively determined in the cell culture medium and lysate. Control virus and Ad-hPON1-infected cells on day 5 were lysed with 250 μl of M-per cell lysis reagent (Pierce Chemical Co., Rockford, IL, USA) and PON1 activity in cell culture medium and lysate was determined using phenyl acetate as the substrate.[18,19]

Partial purification of recombinant hPON1

HEK-293A cells (10×10^6) were seeded in 150 cm^2 tissue culture dishes. After 24 h, the cells were infected with Ad-hPON1 (50 pfu per cell) for 1–2 h using 10 ml of infection medium at 37 °C. To the infected cells, 15 ml of growth medium was added and the cell cultures were incubated for 5–6 days when the activity of hPON1 levels peaked. The spent medium was collected and cleared of cell debris by centrifugation at 2500 rpm for 10 min at 4 °C. Partial purification of recombinant hPON1 was accomplished by subjecting the medium to ammonium sulfate fractionation followed by metal-affinity chromatography. Recombinant hPON1 from 100 ml of the culture medium was precipitated by raising the ammonium sulfate saturation from 50 to 80%. The precipitate was re-suspended and dialyzed against equilibration buffer (50 mM Tris–HCl pH 8.0, 2 mM $CaCl_2$, 0.5 M NaCl). The dialysate was passed through a BD-Talon Cobalt-affinity resin column and the resin-bound recombinant hPON1 was eluted using equilibration buffer containing 500 mM imidazole and identified by western blot analysis.

Enzyme assays

The activity of PON1 was determined by using two substrates, paraoxon and phenylacetate, to confirm that both esters are hydrolyzed by hPON1.

PON1 activity was measured with 1 mM paraoxon in a total volume of 800 μl of 50 mM glycine NaOH, 2 mM $CaCl_2$, pH 10.0.[20] The reaction was initiated by the addition of enzyme. Formation of the product, p-nitrophenol, was recorded as increase in absorbance at 412 nm at room temperature. The amount of p-nitrophenol produced was calculated using the molar extinction coefficient of 18 290 M^{-1} cm^{-1}. Blanks without enzyme were used to correct for

spontaneous hydrolysis of paraoxon. One unit of paraoxonase activity is defined as 1 nmol of p-nitrophenol formed per min under the assay conditions.

PON1 activity measured using phenylacetate as a substrate was determined as described previously.[20] Briefly, the cuvette contained 1 mM phenylacetate in 10 mM Tris–HCl, pH 8.0 and 2 mM $CaCl_2$ in a total volume of 2.0 ml. The reaction was initiated by the addition of enzyme. Formation of the product, phenol, was recorded as increase in absorbance at 270 nm at 25 °C. The amount of phenol was calculated according to the molar extinction coefficient of $1310\,M^{-1}\,cm^{-1}$. Blanks without enzyme were used to correct for spontaneous hydrolysis of phenylacetate. One unit of PON1 activity is defined as 1 μmol phenylacetate hydrolyzed per min under the above assay conditions.

Animal experiments

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adhered to principles stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 Edition. All efforts were made to minimize animal suffering and to reduce the number of animals used for these studies. For these studies, adult female and male mice strain 129 Sv (20–25 g body weight) were housed at 20 °C and were provided food and water *ad libitum*.

Injection of Ad-hPON1 and Ad-control virus. On day 0, blood was collected from the saphenous vein before injection of mice with the recombinant (n=8) or control virus (n=8). Ad-hPON1 or Ad-control viruses were diluted in sterile saline to produce an inoculum of 4×10^{10} pfu in 150 μl of saline. The inoculum was injected rapidly via the tail vein in 5–10 s. Animals were observed for 30 min post-dosing for any untoward signs. Blood was collected for 10 days post-dosing via the saphenous vein into heparinized hematocrit tubes and centrifuged at 14 000 r.p.m. for 10 min at 4 °C. The plasma was removed and assayed for PON1 activity with paraoxon and phenylacetate or stored at −80 °C for use in further studies.

Determination of PON1 levels in the tissue of virus-injected mice. In one experiment, two Ad-hPON1- and one Ad-control virus-injected animals were euthanized on day 4 post-virus injection. The animals were perfused with 50 ml of 0.1 M phosphate-buffered saline before their liver, lung, heart, brain, kidney, muscle, intestine, stomach, skin, testes, spleen, diaphragm, salivary glands and fat were removed and flash frozen. Tissues were homogenized in ice cold buffer containing 50 mM potassium phosphate pH 7.4, 0.5% Tween 20 and centrifuged at 12 000 r.p.m. for 10 min at 4 °C. The supernatants were removed into clean tubes and assayed for PON1 activity with phenylacetate.

Determination of LD_{50} of diazoxon in strain 129Sv mice. Diazoxon (C12H21N2O4P) is the oxon version of pesticide diazenon (C12H21N2O3PS), usually formed in the body by microsomal oxidation of P=S to P=O bond. As LD_{50} values depend on the mouse strain, we determined the LD_{50} of diazoxon by treating adult strain 129 Sv mice with various doses of diazoxon dissolved in ethanol. The LD_{50} value was found to be $14\,mg\,kg^{-1}$ injected subcutaneously.

Challenge with diazoxon. Ad-hPON1 (n=6) and Ad-control (n=3) were injected into the tail vein of mice as described above. Plasma was collected from all the mice before viral injection and daily through day 4 post-viral injection. PON1 activity was assayed with phenylacetate. On day 4 post-viral injection baseline temperature, body weights and observations were recorded. Mice were challenged subcutaneously with $2LD_{50}$ of diazoxon ($28\,mg\,kg^{-1}$). Mice were observed continuously through 1-h post-challenge. Axial body temperatures and signs of cholinergic toxicity were recorded hourly through 7 h post-challenge. Moribund mice were euthanized immediately. Animals that survived the first $2LD_{50}$ challenge were challenged with another $2LD_{50}$ 24 h after the first challenge. The same procedures were followed during the second challenge. Animals that survived $4LD_{50}$ were injected 24 h later with another $2LD_{50}$ following the same procedures.

Sodium dodecyl sulfate–PAGE and western blotting

Sodium dodecyl sulfate–PAGE was carried out with precast 10% gels. After electrophoresis, the proteins were transferred to PVDF membrane. The membrane was blocked in 4% powdered milk for 1 h, washed once with Tris-buffered saline containing Triton X-100 (TTBS) and incubated overnight in primary antibody (anti-6×histidine antibody, 1:1000 dilution or rabbit polyclonal antibody to recombinant hPON1, 1:2000 dilution) in 0.5% milk powder containing 0.01% sodium azide. The membrane was washed with TTBS five times with intermittent shaking for 8 min and incubated with a secondary antibody conjugated with Horse Radish Peroxidase ($100\,ng\,ml^{-1}$) in 0.5% milk powder for 1 h. The membrane was washed again as above and the protein bands detected using ECL-Plus reagent. Chemiluminescence was measured in a Bio-Rad image reader.

Non-denaturing PAGE for esterase activity

PON1 activity in mouse plasma was visualized on non-denaturing polyacrylamide gradient gels (4–30%) stained for esterase activity. Plasma samples were mixed with 10 volumes of 25% glycerol, 0.05 M Tris–Cl, pH 7.5, 0.05% bromophenol blue before loading on the gel. The equivalent of 1 μl of plasma from an Ad-PON1-injected mouse, 1 μl of human serum and 1 μl of wild-type mouse plasma was loaded per lane. Gels were run for 5000 V h at 4 °C. The staining for PON1 activity was adopted from methods described before.[39,40] The staining solution contained 100 ml of 50 mM Tris–Cl, pH 7.4, 10 mM calcium chloride, 50 mg of β-naphthylacetate dissolved in 1 ml of ethanol and 50 mg of solid Fast Blue RR. Undissolved naphthylacetate and Fast Blue RR are present, but do not interfere with color development. Reactive enzyme was identified by pink bands. After 20–60 min, the gel was washed with water; spots of solid reagents were washed off with 50% methanol, 10% acetic acid.

Plasma contains multiple esterases including PON1, BChE, CES and ALB. To distinguish between them, gels were preincubated for 30 min with 0.2 mM DFP or 0.2 mM diazoxon to inhibit BChE and CES, or with 10 mM EDTA to inhibit PON1 before they were stained with β-naphthylacetate and Fast Blue RR. The ALB band was identified by counter-staining the gels with Coomassie blue.

Non-denaturing PAGE for PON1 activity and HDL

A non-denaturing polyacrylamide gel (4–30%, 0.75 mm) loaded with duplicate samples was run for 3200 V h. Mouse plasma (1 μl) with no expressed Ad-hPON1 ($70\,U\,ml^{-1}$) was compared with plasma from mice (1 μl) expressing Ad-hPON1 (2568, 942 and $1656\,U\,ml^{-1}$). Purified hPON1 containing no associated HDL was run as a control (purified hPON1 was a gift from Mrs Frederique Renault and Dr Daniel Rochu, IRBA-CRSSA, La Tronche, France). PON1 activity in the gels was identified by staining one section of the gel with β-napthyl acetate and Fast blue RR. Activity was shown by the presence of diffuse purple bands. The presence of HDL in the samples was detected by staining the gel section overnight in Sudan Black B (Sigma, St Louis, MO, USA), a dye that detects lipoproteins.[41]

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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