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Persistent and high-level expression of human liver prolidase *in vivo* in mice using adenovirus

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ABSTRACT

Human liver prolidase, a metal-dependent dipeptidase, is being tested as a potential catalytic bioscavenger against organophosphorus (OP) chemical warfare nerve agents. The purpose of this study was to determine whether persistent and high-levels of biologically active and intact recombinant human (rHu) prolidase could be introduced *in vivo* in mice using adenovirus (Ad). Here, we report that a single intravenous injection of Ad containing the prolidase gene with a 6× histidine-tag (Ad-prolidase) introduced high-levels of rHu prolidase in the circulation of mice which peaked on days 5–7 at 159 ± 129 U/mL. This level of prolidase is ~120 times greater than that of the enzyme level in mice injected with Ad-null virus. To determine if all of Ad-prolidase-produced rHu prolidase was exported into the circulation, enzyme activity was measured in a variety of tissues. Liver contained the highest levels of rHu prolidase on day 7 (5647 ± 454 U/g) compared to blood or any other tissue. Recombinant Hu prolidase hydrolyzed DFP, a simulant of OP nerve agents, *in vitro*. *In vivo*, prolidase overexpression extended the survival of 4 out of 6 mice by 4–8 h against exposure to two $1 \times LD_{50}$ doses of DFP. In contrast, overexpression of mouse butyrylcholinesterase (BChE), a proven stoichiometric bioscavenger of OP compounds, protected 5 out of 6 mice from DFP lethality and surviving mice showed no symptoms of DFP toxicity. In conclusion, the results suggest that gene delivery using Ad is capable of introducing persistent and high levels of human liver prolidase *in vivo*. The gene-delivered prolidase hydrolyzed DFP *in vitro* but provided only modest protection *in vivo* in mice, delaying the death of the animals by only 4–8 h.

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1. Introduction

A promising approach to mitigating the toxicity of organophosphorus (OP) nerve agents is the use of enzymes that can bind and hydrolyze hundreds and thousands of molecules of nerve agent per each molecule of the enzyme [1,2]. Human (Hu) liver prolidase is one such enzyme that can potentially hydrolyze OP nerve agents *in vivo*. *In vitro* studies, using the enzyme from *Alteromonas undina* and *Alteromonas haloplanktis* have shown that the enzyme hydrolyzes soman, sarin, and diisopropyl fluorophosphate (DFP) [3–5]. Hu prolidase expressed in *Saccharomyces cerevisiae* has also been shown to hydrolyze soman [6,7]. More recently, recombinant (r) Hu prolidase expressed in *Escherichia coli* was found to hydrolyze all four G-type nerve agents *in vitro* [8]. In contrast, rHu skin and kidney prolidases expressed in *Tirchoplusia ni* larva only hydrolyzed DFP and sarin *in vitro* [9]. However, detailed *in vivo* studies

evaluating the efficacy of Hu prolidase to protect animals from OP nerve agent toxicity are lacking. Here, we report that an adenovirus (Ad) containing the gene for Hu prolidase with a 6× histidine-tag at its carboxyl terminus (Ad-prolidase) is capable of producing persistent and high levels of the enzyme *in vivo* in mice. The virus-induced enzyme was structurally intact in mouse blood. Even though, Ad-prolidase-produced rHu prolidase hydrolyzed DFP *in vitro*, overexpression of the enzyme offered meager/modest protection *in vivo* by delaying the time to death of animals by about 4–8 h.

2. Materials and methods

2.1. Recombinant adenoviruses

Ads containing the genes that encode for Hu prolidase and mouse butyrylcholinesterase (BChE) as fusion proteins with a 6× histidine-tag were produced as described before [10,11]. Ad-MoB-ChE was used as a positive control in the challenge experiments using DFP. Ad-null (control) virus was purchased from Welgen Inc., Worcester, MA.

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2.2. Prolidase activity assay

Mouse plasma or tissue samples were tested for prolidase activity by the method of Isaac et al. [12]. Samples were diluted to 100 μ l of 50 mM Tris/HCl, pH 7.4, containing 1 mM MnCl_2 and were preincubated for 24 h at 37 °C. Samples were then mixed with 100 μ l of 94 mM glycyl-proline (Sigma–Aldrich, St. Louis, MO) in 50 mM Tris/HCl, pH 7.4, containing 2 mM MnCl_2 . After 30 min of incubation, the enzyme reaction was stopped with the addition of 1 ml of 0.45 M trichloroacetic acid, and the supernatant was used for proline determination as described previously [12]. One unit of prolidase activity represents the amount of enzyme that catalyzes the hydrolysis of one micromole of substrate, per minute at 37 °C.

2.3. DFP hydrolysis assay

Mouse plasma (5 μ l) or 10% liver homogenates (5 μ l) were added to 3 ml of 50 mM Tris–HCl (pH 7.4) containing 2 mM MnCl_2 and 5.74 mM DFP, and the increase in fluoride ion release was monitored for 10 min using an Orion meter connected to a fluoride ion selective electrode. Five microliters of the same buffer instead of plasma was used as a blank. Activity is reported as U/ml, where 1 U represents 1 μ mole of fluoride ion liberated/min/ml.

2.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting

SDS–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 (monoclonal anti-6 $\times$ histidine antibody, 1:1000 dilution, Cat # 15149, Ab Cam, Cambridge, MA) 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 was incubated with a secondary antibody conjugated with horse radish peroxidase (Cat # Ab6721, Ab Cam, Cambridge, MA; 100 ng/ml) in 0.5% milk powder for 1 h. The membrane was washed again as described above and the protein bands were detected using ECL-Plus reagent. Chemiluminescence was measured in a Bio-Rad image reader.

2.5. Animal studies

Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principle stated in the Guide for the Care and Use of Laboratory Animals, NRC Publication, 1996 edition. Ad-prolidase and Ad-null (2×10^{11} vp/animal) were injected into 6- to 7-week old female Swiss Webster mice through the tail vein. Blood samples were collected on the day before virus injection and daily for 15 days after virus injection and were assayed for Hu prolidase levels by Western blotting and enzymatic activity [12]. In some experiments, three animals were sacrificed on day 4, day 7, day 10 and day 15, and different tissues (i.e., brain, heart, lung, diaphragm, liver, kidney, spleen, and muscle) were collected after saline perfusion and assayed for Hu prolidase levels.

2.6. Human prolidase levels in mouse tissues

Ten percent homogenates of the tissues were made using tissue extraction reagent (T-PER, Pierce Chemical Company) containing protease inhibitor cocktail reagent, and were assayed for Hu prolidase levels by enzyme activity assay.

2.7. Challenge with DFP

Ad-prolidase ($n = 6$, dose of virus = 2×10^{11} vp/animal), Ad-MoBChE ($n = 6$, dose of virus = 2.5×10^{11} vp/animal) and Ad-control ($n = 6$, dose of virus = 2×10^{11} vp/animal) were injected into the tail vein of mice and plasma was collected daily for 5 days. On day 5, mice were challenged intraperitoneally with $1 \times \text{LD}_{50}$ of DFP (6 mg/kg body weight). Mice were observed continuously through 3 h after challenge for signs of cholinergic toxicity. Moribund mice were euthanized immediately. Animals that survived the first $1 \times \text{LD}_{50}$ DFP were challenged with a second LD_{50} of DFP 3 h later and observed for toxic symptoms.

3. Results and discussion

3.1. Time course expression of prolidase in vivo in mice

The ability of Ad-prolidase to induce the expression of full-length rHu prolidase was first confirmed *in vitro* in 293A cells (data not shown). To test whether Ad-prolidase also induces the enzyme expression *in vivo*, Swiss Webster female mice were injected with Ad-prolidase (2×10^{11} viral particles per mouse) via the tail vein. The expression of rHu prolidase was determined in plasma over a 15 day period using glycine-proline (Gly-Pro) as the substrate (Fig. 1). Each time point represents mean $\pm$ SD of 6–8 mice. Two peaks were observed following plotting of the prolidase activity (U/ml) against the day after virus injection: a minor peak on days 2 and 3 and a major peak on days 5, 6, and 7. Overall, prolidase levels were 100- to 150-fold higher (159 ± 129 U/ml) in mice injected with Ad-prolidase than in mice injected with Ad-null virus (1–2 U/ml) on days 5–7. These results demonstrate that a single injection of Ad-prolidase was capable of introducing persistent, high-levels of biologically active rHu prolidase into the systemic circulation of mice.

To determine if Ad-prolidase-produced enzyme was of full-length, plasma from the mouse expressing rHu prolidase at 256 U/ml on day 6 was analyzed by Western blotting with anti-His antibody. The antibody recognized a single 54 kDa band in all

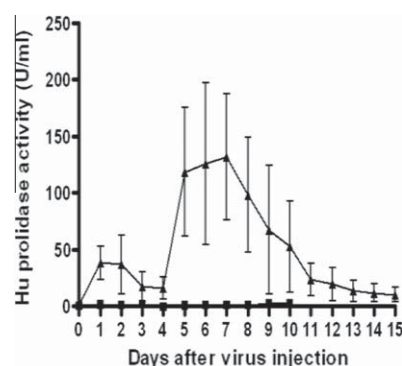


Fig. 1. Expression levels of human liver prolidase in mouse blood following a single tail vein injection of the virus containing the gene for the enzyme. $\blacktriangle$ Ad-prolidase virus, $\blacksquare$ Ad-Null virus.

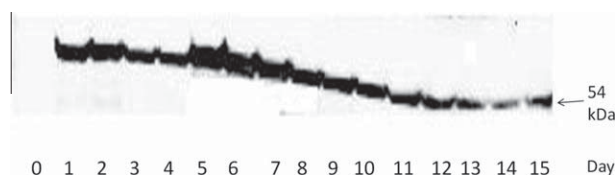


Fig. 2. Western blot of recombinant human prolidase in mouse plasma.

the samples (Fig. 2, days 1 through 15). The pre bleed sample (day 0) did not contain any antibody reactive protein. These results demonstrate that Ad-prolidase expressed enzyme is of full-length and intact in the circulation of mice for as long as 15 days.

3.2. Recombinant human liver prolidase expression in mouse tissues

We determined whether Ad-prolidase-produced enzyme is retained in any of the mouse tissues or fully exported into circulation. On day 4 after virus injection, three Ad-prolidase mice and three Ad-control mice were euthanized for determination of prolidase activity levels in tissues including liver, diaphragm, kidney, brain, lung, heart, spleen and muscle. Liver contained the highest amount of rHu prolidase compared to any other tissue. Prolidase activity of 2487 ± 137 U/g in the livers of mice injected with Ad-prolidase was 200- to 300-fold greater than the activity of the enzyme in the livers of animals injected with the control virus (Fig. 3).

Since liver is the primary tissue of virus activity and contained most of the virus-produced enzyme, prolidase levels were determined in the livers of mice on days 4, 7, 10, and 15 following Ad-prolidase injection (Fig. 4). Recombinant prolidase levels were highest on day 7 (5647 ± 454 U/g tissue) compared to day 4 and day 10 and returned closer to baseline levels on day 15. On day 7, the plasma rHu prolidase levels were 140 ± 56 U/ml, which is 40-fold lower than those in the liver. These results suggest that a large amount of Ad-prolidase-produced enzyme was retained in the liver and was not exported into the circulation.

3.3. Hydrolysis of DFP by recombinant human liver prolidase

Recombinant Hu prolidase was tested for its ability to hydrolyze DFP, a simulant compound of OP nerve agents, by an *in vitro* DFP hydrolysis assay. DFPase activity was undetected in the plasma of mice injected with Ad-control compared to a value of 5.67 ± 2.58 U/ml in the plasma of mice injected with Ad-prolidase on day 4 (Fig. 5, inset). Similarly, DFPase activity in the livers of mice injected with Ad-prolidase was higher by 50-fold on day 4, 90-fold on day 7, 12-fold on day 10 and 10-fold on day 15 than in the livers of mice injected with Ad-control (Fig. 5). These results suggest that Ad-prolidase-produced rHu prolidase is capable of hydrolyzing DFP *in vitro*.

3.4. Recombinant Hu prolidase failed to offer substantial protection against DFP *in vivo*

The most notable nerve agent stoichiometric bioscavenger, BChE, which offers protection against all types of nerve agents

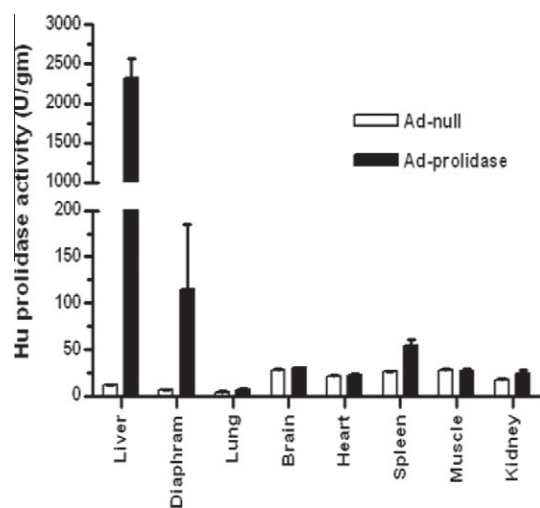


Fig. 3. Expression levels of recombinant human liver prolidase in mouse tissues.

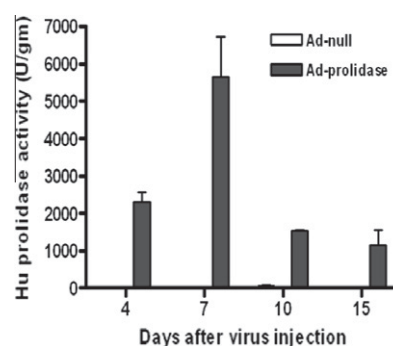


Fig. 4. Time course of recombinant human liver prolidase expression in mouse liver.

and pesticide compounds, is mostly exported into the circulation [11]. Similarly, human paraoxonase1, a promising enzyme in the category of catalytic bioscavengers, is fully found in the circulation [13]. In contrast, gene-delivered rHu prolidase largely remained in the liver (Figs. 3–5), and only a small amount (3–5%) crossed over into plasma. Nevertheless, this amount of rHu prolidase is 50- to 150-fold higher than the prolidase baseline activity in mouse blood. We considered that such high levels of rHu prolidase in mouse plasma would be sufficient to provide protection to the animal, provided that the catalytic activity/efficiency of the enzyme was high enough for the enzyme to hydrolyze the DFP prior to its escape from circulation which leads to cholinergic crisis and eventual death.

First, we determined the LD₅₀ dose of DFP in Swiss-Webster female mice. Six mice were challenged intraperitoneally with one of the three following DFP doses: 6, 9, and 12 mg/kg. Three mice in the 6/mg/kg group, 1 mouse in the 9 mg/kg group, and 0 in the 12 mg/kg group survived the DFP challenge, suggesting that the LD₅₀ dose of DFP for this strain of mice is ~6 mg/kg. This value is consistent with the LD₅₀ values found in other strains of mice [14].

To determine the protection offered by rHu prolidase against DFP toxicity, three groups of mice (Control, Prolidase, and BChE) with 6 animals in each group were injected with Ad-null, Ad-prolidase and Ad-MoBChE, respectively and 5 days later challenged intraperitoneally with $1 \times$ LD₅₀ dose of DFP. Mice injected with Ad-BChE were included as a positive control group since BChE is expected to fully protect against all OP compounds [1,2]. Surviving mice in each group were challenged with a second $1 \times$ LD₅₀ dose of DFP three hours after the first challenge (Table 1). Four of 6 animals in the control group, and 6 of 6 animals in the prolidase and BChE groups survived the first LD₅₀ dose of the toxicant. The second LD₅₀ injection of DFP 3 h later resulted in the death of all the animals in the control group within 15 min of the toxicant injection. By contrast, only 2 of the 6 animals in the prolidase group and 1 of the 6 animals in the BChE group died. In the prolidase group, the 4 surviving mice demonstrated signs of nerve agent poisoning, including piloerection, salivation, and splayed hind limbs, etc. These four mice were found dead the following morning, suggesting that prolidase overexpression failed to offer protection against DFP *in vivo*. In contrast, the 5 mice in the BChE group showed no DFP toxicity symptoms at 24 h. The one animal death in the BChE group may have been the result of an inadequate amount of BChE expression in its blood because it did not receive a sufficient amount of Ad-BChE. Taken together, these data suggest that rHu prolidase, at best, offered meager protection against DFP toxicity *in vivo* by extending the survival of 4 out of 6 mice by about 4–8 h. The lack of human liver prolidase to offer protection against DFP *in vivo* in mice may not be due to inadequate enzyme activity. The enzyme to toxicant ratio on the day of toxicant challenge was 1:100–200

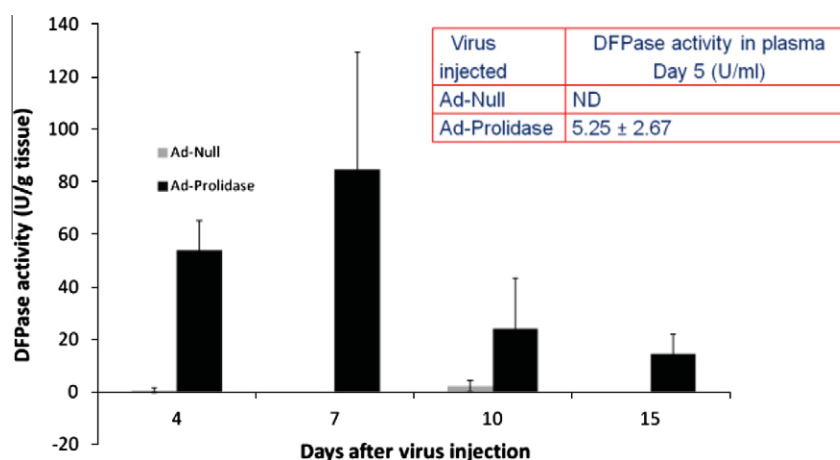


Fig. 5. Hydrolysis of DFP by recombinant Hu liver prolidase expressed in mouse liver (Bar graph) and plasma (inset).

Table 1

Group	DFP challenge 1st (^a animals)	Survival	DFP challenge 2nd	Overnight survival after the challenge
Ad-Control	6	4/6	4	0/4
Ad-Prolidase	6	6/6	6	0 ^a /6
Ad-MoBChE	6	6/6	6	5 ^b /6

^a Unlike Ad-control animals which died 15 min after the 2nd DFP challenge, Ad-prolidase injected animals took 4–8 h to die.

^b Mice injected with MoBChE expressing virus were alive 24 h after the 2nd DFP challenge without any signs of DFP poisoning.

(4–8 nmoles of the enzyme vs 800 nmoles of DFP). Most likely, the catalytic efficiency of recombinant human liver prolidase is not high enough to rapidly hydrolyze DFP and lower its blood concentration to non-lethal levels. A similar conclusion has been drawn based on its *in vitro* catalytic efficiency for the enzyme expressed and purified from *E. coli* [8]. Studies evaluating the ability of rHu prolidase to mitigate the toxicity of nerve agents, if any, are ongoing.

4. Conclusions

Taken together, the results suggest that Ad type 5 is useful to deliver high-levels of intact and functional rHu liver prolidase *in vivo* in mouse blood. Compared to mice injected with a control virus, the mice injected with Ad containing the gene for Hu liver prolidase contained 50- to 150-folds higher levels of the enzyme on day 6 in their blood. High-levels of the enzyme remained in mouse blood for 2–3 days. Most of the enzyme produced via Ad remained in mouse liver. Recombinant Hu prolidase hydrolyzed DFP *in vitro*, but failed to offer substantial protection *in vivo* in mice, only delaying the death of mice by about 4–8 h.

Disclaimer

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The experimental protocol was approved by the Animal Care and Use Committee of the US Army Medical Research Institute of Chemical Defense (Aberdeen Proving Ground, MD) and all procedures were conducted in accordance with the principles stated in

the Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act of 1966 (P.L. 89-544), as amended.

Conflict of interest statement

None declared.

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