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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× 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 *Alteromonos 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-MoBChE 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 µl 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 µl 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 µl) or 10% liver homogenates (5 µl) 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 µmole 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× 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× 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× 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 ± 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

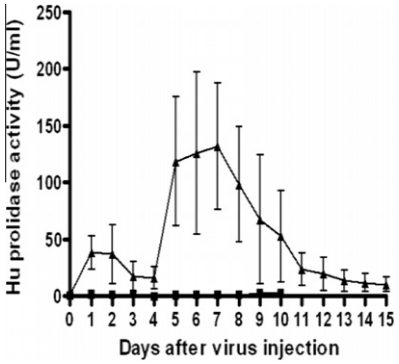

Fig. 1. Expression levels of human liver prolidase in mouse blood following a single tail vein injection of the virus containing the gene for the enzyme. ▲ Ad-prolidase virus, ■ Ad-Null virus.

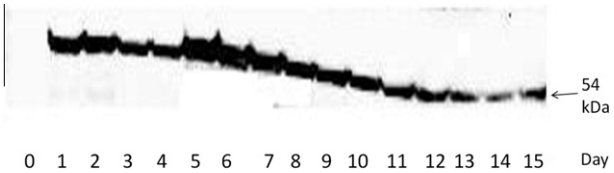

Fig. 2. Western blot of recombinant human prolidase in mouse plasma.

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