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Overexpression of Human Senescence Marker Protein 30 in Mice Fails to Offer Protection against Challenge with Organophosphorus Compounds

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Abstract

To investigate the ability of human senescence marker protein 30 (SMP30) to offer protection against chemical warfare nerve agents *in vivo*, we produced a recombinant human SMP30 fusion protein with a hemagglutinin tag expressed using an adenovirus (AD-SMP30). A single intravenous injection of Ad-SMP30 into mice transduced the expression of SMP30 fusion protein in a dose and time dependant manner in the liver and diaphragm, but not in the plasma or other tissues examined in mice. Expression of SMP30 in liver and diaphragm was noted on day 2, reached peak levels on day 4 and declined thereafter. The expression levels of SMP30 achieved in the liver of Ad-SMP30-injected mice compared to the liver of mice injected with an Ad-null virus was ~10- to 25-fold higher on day 4. Despite these high levels of SMP30, mice challenged with supra lethal doses of diisopropylfluorophosphate, VX or soman did not gain any discernible protection. Time to death between the control animals and animals with elevated levels of human SMP30 was not significantly different. These results suggest that wild-type human SMP30 expressed in the tissues of mice failed to offer protection against lethal levels of organophosphorus compounds *in vivo*.

1. Introduction

Organophosphorus nerve agents (OPs) present a serious life-threatening risk to both soldiers and civilians [1]. Current antidotal regimen for OP poisoning includes anticholinergic drugs to counteract the effects of elevated acetylcholine, oximes to reactivate acetylcholinesterase (AChE) and anticonvulsants to control OP-induced symptoms of tremors and convulsions [2]. However, these treatments have undesirable side effects, require administration within a prescribed time frame for maximal efficacy and are unable to completely prevent post-exposure complications [3]. Hence, a pre-treatment regimen for OP exposure scenarios is highly desirable and is being actively pursued.

One approach under investigation is the development of human proteins as bioscavengers that sequester or hydrolyze OPs before they reach and inhibit AChE [4,5]. Currently, plasma-derived human butyrylcholinesterase (BChE) and Protexia™, a recombinant BChE produced in the milk of transgenic goats, are promising [6,7]. However, BChE is a stoichiometric bioscavenger, thus requiring large quantities (200 mg/70 Kg person) of the enzyme to provide protection against 2x LD₅₀ of soman [8]. More recently, enzymes that can inactivate OPs without being consumed are being investigated for their potential as catalytic bioscavengers [9]. 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. Human senescence marker protein 30 (SMP30) has the potential to be such a candidate.

SMP30 is a 34 kDa protein first isolated from rat liver. The level of SMP30 decreases with age in an androgen independent manner suggesting its possible roles in age-related physiologic and pathologic conditions [10,11,12,13]. SMP30 is widely distributed in different vertebrate tissues including liver, kidney, brain, testis, lungs, adrenal gland, stomach, ovary, uterus and epidermis [13]. Immunohistochemical and Western blot analyses show that SMP30 is localized in the cytosol and nucleus of hepatocytes [14], and in kidneys, it was primarily in renal proximal tubular epithelia [11].

SMP30, also known as regucalcin, is reported to have multifunctional physiological roles. One of the major roles described for SMP30 is in maintaining Ca²⁺ homeostasis by activating enzymes involved in the regulation of Ca²⁺ pumps localized in the plasma membrane, microsomes and mitochondria of different cell types [15]. SMP30 can bind to Ca²⁺ even though it lacks commonly known Ca²⁺ binding motifs such as the EF-hand [16]. In the nucleus, SMP30 is believed to be involved in the regulation of protein kinases, protein phosphatases, and deoxyribonucleic acid and ribonucleic acid biosynthesis [17]. Over-expression of SMP30 in rats leads to osteoporosis [17] and hyperlipidemia [18], while SMP30 deficiency in mice causes accumulation of neutral lipids and other phospholipids in the liver [19], demonstrating its critical roles in bone and lipid metabolism. Studies conducted using SMP30 knock-out mice indicate that brain SMP30 has a protective role against oxidative damage without influencing the enzymes involved in antioxidant protection [20]. The enzyme also plays an important role in ascorbic acid biosynthesis in the liver of vertebrates [21].

The amino acid sequence of mouse SMP30 was found to be identical to an enzyme isolated from rat liver having the ability to hydrolyze diisopropylfluorophosphate (DFP) and nerve agents like sarin, soman and tabun [22]. Moreover, the liver of SMP30 knock-out mice lacked DFPase activity, which suggests that SMP30 may be the DFP hydrolyzing enzyme, and hence it could be a potent bioscavenger against OPs like the chemical warfare nerve agents (CWNA) [23]. Even

though there are structural similarities between SMP30 and serum paraoxonase 1 (PON1), another promising catalytic bioscavenger, the inability of SMP30 to hydrolyze PON1 specific substrates makes SMP30 distinct from the PON1 family lineage [23]. The crystal structure of human SMP30 and the catalytic role of the metal ion have been recently elucidated [24].

In this study, our goal was to investigate the *in vivo* efficacy of human SMP30 in the detoxification of CWNA and simulants by overexpressing SMP30 through adenovirus-mediated gene delivery approach. Earlier, we demonstrated that this approach is capable of introducing clinically relevant amounts of mouse BChE and human PON1 in mice which were protected from 5x LD₅₀ doses of the nerve agents VX and soman and 2-6x LD₅₀ doses of diazoxon [25,26]. We have made recombinant virus encoding human SMP30 as a fusion protein with a hemagglutinin (HA) tag at its carboxyl terminus (Ad-SMP30) and expressed human SMP30 in mice by a single tail vein injection of Ad-SMP30. We report that mice with 10- to 25-fold elevated levels of human SMP30-HA fusion protein in liver and diaphragm were not protected from lethal doses of DFP, VX and soman. These results suggest that wild-type human SMP30 fused to HA and expressed in mouse liver and diaphragm failed to offer protection against CWNA and is not promising in its wild-type form for development as a bioscavenger.

2. Materials and Methods

2.1 Materials

Nerve agents *O*-pinacolyl methylphosphonofluoridate (soman) and *O*-ethyl *S*-2-*N,N*-diisopropylamino ethyl methylphosphonothioate (VX) were obtained from the US Army Edgewood Chemical Biological Center (ECBC), Aberdeen Proving Ground, MD. The purity of soman and VX was >98.5% as determined by ³¹P-NMR. Sigma-Aldrich (St. Louis, MO) was the source for DFP. Other chemicals and reagents were of highest purity available.

2.2 Production of recombinant adenoviruses

Construction and characterization of Ad-SMP30 and adenovirus expressing truncated human BChE (Ad-HuBChE) and preparation of a high titer viruses were carried out as described earlier [27, 28]. Control virus lacking SMP30 or HuBChE but otherwise identical (Ad-null) was purchased from Welgen Inc. (Worcester, MA).

2.3 Dose response and time course expression of human SMP30 in mice

The experimental protocol was approved by the Animal Care and Use Committee at the United States Army Medical Research Institute of Chemical Defense, 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. All efforts were made to minimize animal suffering and to reduce the number of animals used in the studies. For these studies, adult female Swiss Webster mice (6-7 weeks old; 20–25 gm body weight) were housed at 20⁰C and were provided food and water *ad libitum*. In dose response experiments, mice (n=4 per group) were administered Ad-SMP30 or Ad-null (low dose; 0.5×10¹¹ or high dose; 2.0×10¹¹ viral particles per animal) intravenously through the tail vein. The animals were sacrificed on day 4, and SMP30 expression was determined in the liver and diaphragm by SDS-PAGE followed by Western blotting using anti-HA and anti-SMP30 antibodies. In time course expression experiments, mice (n=10 for Ad-SMP30) were administered Ad-SMP30 or Ad-null (2.0×10¹¹ viral particles per animal) intravenously through the tail vein and were

sacrificed on days 2, 4, 6, 8 and 10 post-administration of the virus to collect plasma, liver, diaphragm, brain, lungs, kidney, spleen and heart samples. Tissues were removed after collecting blood in heparin followed by saline perfusion.

2.4 Challenge of mice with OPs

Mice were given Ad-SMP30, Ad-null (negative control), or Ad-HuBChE (positive control) (2.0×10^{11} viral particles per animal) intravenously through the tail vein. On day 4, animals were challenged with DFP (intraperitoneally, 6 mg/kg, 1x LD₅₀ dose; orally 45 mg/kg, 1.5x LD₅₀ dose), or VX (subcutaneously, 21 µg/kg, 1x LD₅₀) or soman 1x LD₅₀ doses (subcutaneously, 110 µg/kg, 1x LD₅₀) and post-exposure symptoms and mortality were monitored. Following the first dose of DFP or nerve agents, surviving animals were given a second 1x LD₅₀ dose of the same compound and post-exposure symptoms and mortality were followed. The BChE group of animals in the soman study received four sequential 1x LD₅₀ doses of soman administered 1 hour apart. All animals in the VX study were challenged with two sequential 1x LD₅₀ doses of VX given 1 hour apart.

2.5 Expression of SMP30 in the plasma of mice injected with Ad-SMP30

To determine whether the plasma from mice injected with Ad-SMP30 on day 4 contains recombinant human SMP30, we took advantage of the HA-tag and performed HA-affinity chromatography. Fifteen µl of plasma was diluted with 500 µl of 20 mM Tris-HCl buffer (pH 7.5) containing 0.1M NaCl and 0.1 mM EDTA, mixed with EZ View Red HA-affinity beads (100 µl, Sigma-Aldrich, St. Louis, MO) and gently mixed for 1 hour. The beads were collected by centrifugation at 2500×g for 2 minutes and washed thrice with the above buffer. In order to check the efficiency of HA-affinity purification, HEK-293 cell extract containing recombinant human SMP30 was added to 15 µl of plasma from mice injected with Ad-null virus and subjected to affinity purification as above. The beads were extracted with 1x sodium dodecyl sulfate (SDS) gel loading buffer containing 5% β-mercaptoethanol by incubating at 95°C for 10 minutes and subjected to Western blotting.

2.6 Expression of SMP30 in tissues

A 10% homogenate of tissues was made in T-Per tissue protein isolation buffer (Pierce Chemical Co., Rockford, IL) containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Total protein was measured using Bio-Rad DC protein assay kit and equal amount of total protein were used for Western blotting.

2.7. Western blotting

SDS-PAGE was carried out with precast 10% Tris-HCl gels. After electrophoresis, the proteins were transferred to PVDF membrane (GE Healthcare, Piscataway, NJ) using a Bio-Rad transfer apparatus. The membrane was blocked in 4% powdered milk for 1 hour, washed once with Tris buffered saline containing 0.05% Triton X100 (TTBS) buffer and kept overnight in primary antibody made in 0.5% powdered milk containing 0.01% sodium azide. The primary antibodies used were either rabbit-anti-HA polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, 1:1000 dilution) or rabbit-anti-SMP30 polyclonal antibody (Cosmo Bio Co., Denver, CO, 1:1000 dilution). The membrane was then washed with TTBS five times with intermittent shaking for 8 minutes and incubated with secondary antibody made in 0.5% powdered milk for 1 hour. The secondary antibody used was goat-anti-rabbit conjugated with horse radish peroxidase (KPL, Inc., Rockville, MD; 1:2500 dilution). The membrane was washed again as above and the

protein bands were detected using ECL-Plus Western blot detecting reagent (GE Healthcare, Piscataway, NJ), and the chemiluminescence was measured in a Bio-Rad image reader.

3 Results and Discussion

3.1 Dose response of Ad-SMP30 in mouse liver and diaphragm

We previously reported a very high level of expression of SMP30-HA fusion protein in HEK-293 cells and in C3A liver cells using Ad-SMP30, suggesting that the virus was biologically active *in vitro* [27]. The same Ad-SMP30 construct was tested for SMP30 expression in mice. Figure 1 shows the dose response of Ad-SMP30 on expression of SMP30 in the liver and diaphragm of two individual mice (n=2 per dose) on day 4 after a single intravenous administration of the virus. The results indicate that Ad-SMP30 transduced the expression of SMP30 fusion protein in the liver and diaphragm in a dose dependant manner. Expression of the SMP30 fusion protein was much higher in liver compared to diaphragm in both the mice. No detectable amount of SMP30 was present in other tissues examined such as brain, heart, kidney, lung, and spleen (results not shown). Comparable results were obtained when the Western blotting was performed with anti-SMP30 antibodies (data not shown). As observed in our earlier studies [27], two smaller molecular weight proteins (~28 and 24 kDa) were also detected in mice infected with Ad-SMP30 but not Ad-Null. These forms were particularly visible at high expression levels in liver extracts of one of the mice injected with Ad-SMP30 (Figure 1). We postulated these smaller bands to be processed forms of the full length 34 kDa SMP30 [27].

3.2 Time course of SMP30 expression

Following the introduction of 2.0×10^{11} Ad-SMP30 viral particles into mice via the tail vein, the expression of SMP30-HA fusion protein was determined over a period of 10 days in liver and diaphragm. Figure 2 depicts expression of SMP30 in these tissues from two mice at each time point. The results suggest that SMP30-HA fusion protein expression has accumulated by day 2, reaches peak levels on day 4, and decreases gradually thereafter. As noted before, expression of SMP30 was significantly higher in liver compared to diaphragm. We estimate that human SMP30-HA fusion protein levels in the liver and diaphragm from Ad-SMP30 mice compared to the liver and diaphragm from Ad-null controls was ~10- to 25-fold higher on day 4. This estimation is based upon comparing the grain intensity of anti-HA antibody reactive of 34 kDa, 28 kDa, and 24 kDa proteins in the liver and diaphragm of Ad-SMP30 injected mice to those from Ad-null virus injected mice. No gross toxicity at these levels of SMP30-HA expression was noted in mice over the 10-day period.

3.3 Expression of SMP30 in the plasma

We determined whether Ad-SMP30 synthesized SMP30-HA fusion protein to be exported into mouse plasma. As shown in Figure 3, we were not able to identify any SMP30 in 2 μ l of plasma from Ad-null or Ad-SMP30 injected mice. To rule out the possibility that the sensitivity of the assay is insufficient with 2 μ l of mouse plasma, we employed HA-affinity chromatography and Western blotting using 15 μ l of mouse plasma from Ad-SMP30 animals. We also performed HA-affinity chromatography and Western blotting on control mouse plasma spiked with 10 μ l of HEK-293 cell extract containing human SMP30-HA as a positive control. While we were able to isolate and readily identify human SMP30 in the control mouse plasma spiked with HEK-293A cell extract, we were not able to detect any SMP30 in 15 μ l of Ad-SMP30 mouse plasma (results not shown but are very similar to the results shown in Figure 3). These results suggest that Ad-

SMP30 transduced human SMP30-HA protein remains as an intracellular protein and is unlikely to be exported into the circulation. These results are in agreement with *in vitro* data in which SMP30 transduced from Ad-SMP30 in HEK293 cells and C3A liver cells remained intracellular and did not secrete into the culture medium [27].

3.4 SMP30 expression did not offer protection against DFP

Endogenous BChE and PON1, which offer protection against nerve agents and certain pesticide compounds, are exported into circulation [25,26]. In contrast, virally expressed human SMP30 fusion protein remained in the liver and diaphragm (Figures 1 and 2) and did not cross over into the plasma (Figure 3). We considered that the high expression levels of human SMP30-HA fusion protein (10- to 25-fold higher than controls) in liver and diaphragm were significant enough to warrant an investigation to determine if such an overexpression of human SMP30 in mice would offer protection against DFP challenge. Mice were challenged with DFP on day 4 after Ad-SMP30 injection. Initial experiments were conducted using intraperitoneal (IP) dosing of the challenge agent. Two consecutive 1x LD₅₀ doses of DFP were administered 3 hours apart to groups of 6 mice (Table 1). Five of 6 animals given Ad-null and 6 of 6 animals given Ad-SMP30 died after the second LD₅₀ dose of DFP. The median elapsed time of death following the second IP dose was ~8-12 min in both control and Ad-SMP30 groups.

We reasoned that the lack of protection against DFP challenge on IP dosing may be related to low or non-existent levels of SMP30 in systemic circulation. We considered that an oral (PO) dosing regimen may be more appropriate given the first pass effect through the liver prior to systemic circulation. The animals received a 1.5x LD₅₀ dose of DFP PO on day 5 following virus injections. All animals (6/6) in both the Ad-null and Ad-SMP30 groups died within a few minutes of DFP administration (Table 1). Collectively, these results suggest that SMP30 overexpression in the liver and diaphragm of mice offered no protection against lethal doses of DFP given IP or PO. We also tested the liver extracts of all six Ad-SMP30 group mice following their death after the second IP LD₅₀ dose for human SMP30 levels by Western blotting. The results, shown in Figure 4, indicate that all the animals that were given Ad-SMP30 and died due to DFP challenge contained elevated levels of human SMP30-HA fusion protein.

3.5 Human SMP30 expressed in liver and diaphragm failed to offer protection against VX and soman

The activity of human SMP30 against VX and soman toxicity was also evaluated in mice. The animals received Ad-null virus, Ad-SMP30 virus or Ad-HuBChE virus (2×10^{11} viral particles per animal) and four days later challenged with sequential 1x LD₅₀ doses of VX (21 µg/kg) or soman (110 µg/kg) given subcutaneously (SC) 1 hour apart. Both VX and soman were equally toxic to animals injected with both Ad-null and Ad-SMP30 viruses (Table 2), with the exception of animal #5 in the Ad-SMP30 group, suggesting that SMP30 overexpression in liver and diaphragm did not offer protection. Animal #5 died immediately following the second soman injection, suggesting that the first soman injection may not be fully successful. In contrast, animals that received the virus responsible for expression of HuBChE [28], a known stoichiometric bioscavenger, survived two sequential LD₅₀ doses of VX or four sequential LD₅₀ doses of soman, and showed no toxicity symptoms for the 24-hour observation period post-challenge.

Despite achieving 10- to 25-fold overexpression of human SMP30 in mice, these animals challenged with toxic LD₅₀ doses of nerve agents and simulant compounds did not get any protection. It is unlikely that the negative results are due to the adenovirus system since we successfully used the same system to demonstrate overexpression of human PON1 and mouse and human BChE as fusion proteins with a 6x histidine tag at their carboxyl terminus in mice and achieved protection against nerve agents and OP compounds [25,26]. Potential explanations are that 1) the organophosphatase activity and catalytic efficiency of human SMP30 are below the threshold levels required to neutralize DFP or nerve agents and 2) lack of sufficient levels of human SMP30 in systemic circulation prevented access of the scavenger to the compartment where OP compounds are likely to accumulate before having deleterious effects on the body. Based on these results, we conclude that wild-type human SMP30 may not be suitable for development as a bioscavenger of CWNA. Similar conclusions were drawn by others based on rates of *in vitro* hydrolysis of G-type nerve agents by purified mouse liver SMP30 [29].

Table 1: Toxicity of IP or PO administered DFP in mice injected with Ad-null and Ad-SMP30 viruses.

| Mouse | Viral construct | Time to death (min) after 2 nd 1x LD ₅₀ DFP, IP | Time to death (min) after 1.5x LD ₅₀ DFP, PO |
|-------|-----------------|---|---|
| 1 | Ad-Null | 107 | 15 |
| 2 | | n/a* | 8 |
| 3 | | 2 | 12 |
| 4 | | 51 | 19 |
| 5 | | 8 | 7 |
| 6 | | 6 | 7 |
| 1 | Ad-SMP30 | 8 | 6 |
| 2 | | 41 | 13 |
| 3 | | 43 | 7 |
| 4 | | 10 | 8 |
| 5 | | 8 | 4 |
| 6 | | 15 | 6 |

*This animal survived the 2nd 1x LD₅₀ dose of DFP and was euthanized as per the protocol after the 1-hour observation period.

Table 2. Nerve agent toxicity and lethality in control and SMP30 overexpressing mice.

| | Soman study* | | | VX study** | | |
|---------------|--------------|--|---|------------|--|---|
| | Animal # | Time to convulsions after agent injection (min) | Time to death after agent injection (min) | Animal # | Time to convulsions after agent injection (min) | Time to death after agent injection (min) |
| Control group | 1 | 1 | 2 | 1 | 4 | 8 |
| | 2 | 1 | 2 | 2 | 5 | 8 |
| | 3 | 1 | 3 | 3 | 17 | 18 |
| | 4 | 2 | 7 | 4 | 4 | 9 |
| | 5 | 1 | 3 | 5 | 7 | 10 |
| | 6 | 1 | 2 | 6 | 6 | 9 |
| SMP30 group | 1 | 3 | 4 | 1 | 9 | 20 |
| | 2 | 2 | 10 | 2 | 4 | 10 |
| | 3 | 2 | 2 | 3 | 5 | 10 |
| | 4 | 2 | 3 | 4 | 3 | 6 |
| | 5 | 63 | 1 | 5 | 2 | 8 |
| | 6 | 2 | 2 | 6 | 9 | 10 |
| BChE group | 1 | No convulsions or any other cholinergic symptoms | No deaths or symptoms over 24 h following the last dose | 1 | No convulsions or any other cholinergic symptoms | No deaths or symptoms over 24 h following the last dose |
| | 2 | | | 2 | | |
| | 3 | | | 3 | | |
| | 4 | | | 4 | | |
| | 5 | | | | | |
| | 6 | | | | | |

* Control group and SMP30 group animals, except for one, in the soman study were challenged with 1x LD₅₀ dose (110 µg/kg) of soman given SC; One animal (#5) in the SMP30 group received a second 1x LD₅₀ dose of soman 1 hour after the first; this animal died immediately after the second soman injection. The HuBChE group of animals in the soman study received four sequential 1x LD₅₀ doses of soman administered 1 hour apart. ** All animals in the VX study were challenged with two sequential 1x LD₅₀ doses (21 µg/kg) of VX given SC 1 hour apart. Blood BChE levels in the BChE group animals prior to the nerve agent challenge were 962 ± 114 U/ml and 984 ± 124 U/ml in VX and soman studies, respectively.

Figure 1

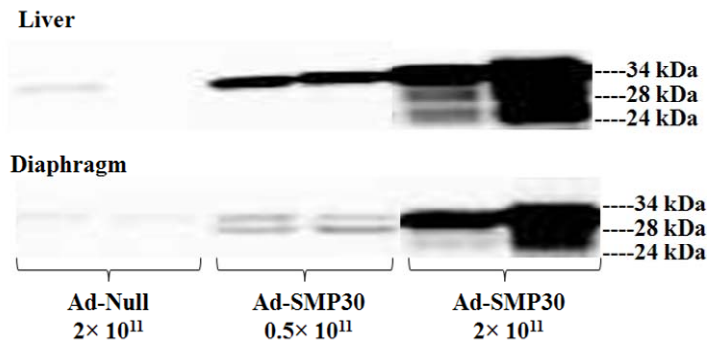


Figure 1: Dose response of Ad-SMP30 in the liver and diaphragm of mice. Animals (n=2 per group) were given a single intravenous injection of Ad-null (2×10^{11} viral particles per animal) or Ad-SMP30 (0.5×10^{11} or 2×10^{11} viral particles per animal) and sacrificed on day 4 after virus injection. Equal amount of total protein was used for SDS-PAGE and Western blotting for determining the SMP30 expression levels using anti-HA tag antibody.

Figure 2

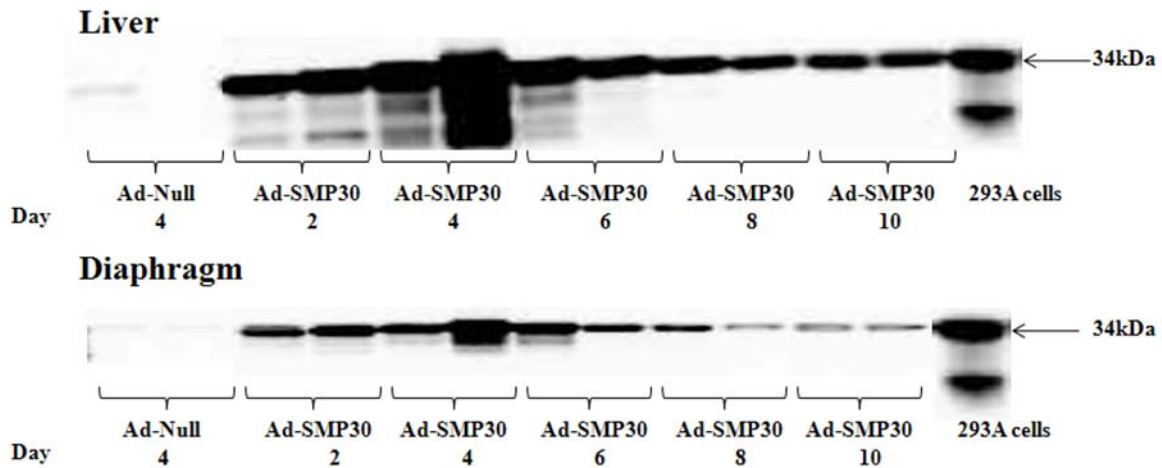


Figure 2: Time course expression of SMP30 in the liver and diaphragm of mice. Animals were given a single intravenous injection of Ad-null (n=2) or Ad-SMP30 (n=2 per time point; 2×10^{11} viral particles per animal). Animals given Ad-null were sacrificed on day 4, and animals given Ad-SMP30 were sacrificed on days 2, 4, 6, 8 and 10 after virus injection. Equal amount of total protein was used for SDS-PAGE and Western blotting using anti-HA antibody. HEK-293 cell extract containing the intact 34 kDa, and the smaller forms of SMP30 was used a positive control for the Western blot (lane marked 293A cells).

Figure 3

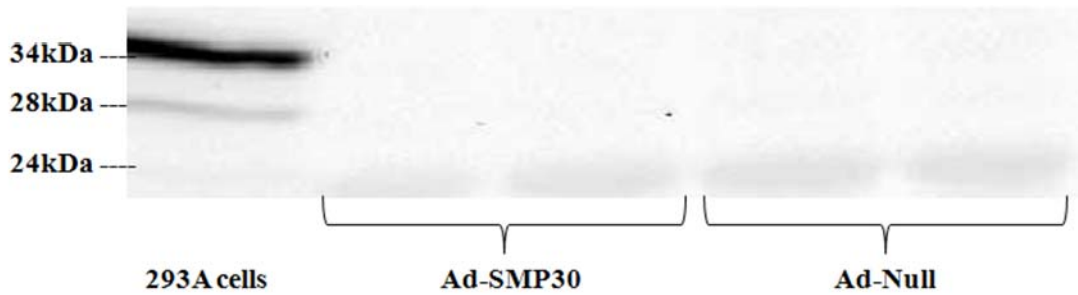


Figure 3: SMP30 expression in the plasma of mice injected with Ad-SMP30. Plasma (2 μ l) from mice injected with Ad-null or Ad-SMP30 (2×10^{11} viral particles) was subjected to SDS-PAGE and Western blotting using anti-HA tag antibody for SMP30 expression. HEK-293A cells expressed SMP30, and its processed forms were used as positive control (lane marked 293A cells).

Figure 4



Figure 4: SMP30 expression in the liver extracts of Ad-SMP30 group mice following DFP challenge. Western blot analysis shows the overexpression of SMP30 in the Ad-SMP30 group of mice which did not survive the second DFP IP challenge

4. Conclusions

In this study, recombinant adenovirus containing the gene for human SMP30 (Ad-SMP30) was tested for its ability to offer protection against supra lethal doses of DFP and the nerve agents soman and VX. Mice injected with Ad-SMP30 intravenously contained 10- to 25-fold higher levels of human SMP30 in their liver and diaphragm compared to the liver and diaphragm from Ad-null virus injected mice. Despite the presence of such high levels of SMP30, mice were not protected from the toxic symptoms or lethality of DFP, VX, and soman. These results suggest that wild-type SMP30 fails to offer protection against the toxicity of OP nerve agents and is not promising in its present form as a bioscavenger of CWNAs.

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