Using Pulmozyme DNase Treatment in Lentiviral Vector Production

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Abstract

In the production of lentiviral vector for clinical studies the purity of the final product is of vital importance. To remove plasmid and producer cell line DNA, investigators have incubated the vector product with Benzonase, a bacterially derived DNase. As an alternative we investigated the use of Pulmozyme, a U.S. Food and Drug Administration-approved human DNase for the treatment of cystic fibrosis, by comparing the efficiency of DNA removal from lentiviral vector preparations. A green fluorescent protein-expressing lentiviral vector was prepared by transient calcium phosphate transfection of HEK 293T cells and DNA removal was compared when treating vector after harvest or immediately after transfection. The effectiveness of DNase treatment was measured by quantitative PCR using primers for vesicular stomatitis virus glycoprotein G viral envelope plasmid. When treating the final product, 1-hr incubations (37°C) with Pulmozyme at 20 U/ml reduced plasmid DNA to undetectable levels. Longer incubations (up to 4 hr) did not improve DNA removal at lower concentrations and the effectiveness was equivalent to or better than Benzonase at 50 U/ml. Attempting to use Pulmozyme immediately after transfection, but before final medium change, as a means to decrease Pulmozyme concentration in the final product provided a 2-log reduction in DNA but was inferior to treatment at the end of production. Pulmozyme, at concentrations up to 100 U/ml, had no measurable effect on infectious titer of the final vector product. The use of Pulmozyme is likely to increase the cost of DNase treatment when preparing vector product and should be considered when generating clinical-grade vector products.

Introduction

LENTIVIRAL VECTORS have shown promise as a new therapeutic tool for patients with life-threatening disorders (Levine *et al.*, 2006; Cartier *et al.*, 2009; Cavazzana-Calvo *et al.*, 2010; DiGiusto *et al.*, 2010; Kalos *et al.*, 2011). Most investigators generating lentiviral vectors use a transient transfection when manufacturing these vectors for research and clinical applications (Dull *et al.*, 1998; Gasmi *et al.*, 1999; Lu *et al.*, 2004). Vector components are separated into different plasmids to reduce the risk of recombination and the potential production of a replication-competent lentivirus (RCL) (Dull *et al.*, 1998; Zufferey *et al.*, 1998), a strategy that to date appears effective (Cornetta *et al.*, 2011). A concern with the transient transfection method is the residual plasmid DNA remaining in the final product, potentially exposing patients to plasmids expressing HIV-1 DNA (Zufferey, 2002). To decrease this risk, the DNase Benzonase has been shown to significantly decrease plasmid DNA in lentiviral vector preparations (Sastry *et al.*, 2004). Unfortunately,

Benzonase is a bacterial-derived product and does not have U.S. food and Drug Administration (FDA) approval, a factor that will be problematic as vectors move from early-phase studies into licensed products. In contrast, Pulmozyme is a recombinant human DNase (rhDNase) that is FDA approved for the treatment of cystic fibrosis (Shak *et al.*, 1990; Shire, 1996; Ulmer *et al.*, 1996; Pan *et al.*, 2001) and could serve as a safer alternative.

The timing of DNase treatment is also an area for potential improvement. DNase is generally added to the vector product after the final harvest, increasing processing time that can decrease the potency of the final product. Moreover, the DNase will remain in the final product unless additional processing is performed. We therefore compared DNase treatment of the final product versus adding DNase earlier in the production process as a potential means to minimize residual DNase in the final product. Our findings demonstrate that Pulmozyme is an effective DNase and a suitable alternative to Benzonase for removing plasmid DNA from lentiviral vector products.

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Materials and Methods

Cell culture and reagents

HEK 293T cell lines were used for vector production and HEK-293 cells were used for transduction and subsequent infectious titer measurement. Both cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in D10 medium (88% high-glucose Dulbecco's modified Eagle's medium [DMEM; Invitrogen, Carlsbad, CA], 10% fetal bovine serum [FBS; HyClone, Logan, UT], penicillin-streptomycin [100 units/ml; Invitrogen], and 2 mM Glutamax [Invitrogen]) incubated at 37°C with 5% CO₂. Viral supernatants were harvested in OptiPRO serum-free medium (Invitrogen).

Vector production

Vector was generated in HEK 293T cells plated at 2×10^6 /T25 flask in D10 medium. After approximately 24 hr the medium was changed to fresh D10 with the subsequent addition of the transfection mixture, using a ProFection mammalian transfection kit (Promega, Madison, WI) according to the manufacturer's instructions. The transgene vector plasmid used was pcDNA-CS-CGW (4.4 µg/T25 flask) provided by P. Zoltick (Children's Hospital, Philadelphia, Pa), carrying the enhanced green fluorescent protein (eGFP) gene driven by the cytomegalovirus (CMV) promoter. The three packaging plasmids used were pMDL expressing Gag-Pol (2.2 µg/T25 flask), pMDG expressing the vesicular stomatitis virus (VSV)-G envelope glycoprotein (1.5 µg/T25 flask), and pRSV-REV for Rev expression (1.1 µg/T25 flask) (Dull *et al.*, 1998; Zufferey *et al.*, 1998). The

cells were then incubated at 37°C for 16–18 hr, after which time the transfection medium was replaced with OptiPRO serum-free medium. Twenty-four hours later the viral supernatant was collected and clarified by passage through a 0.45-µm filter and stored at –80°C.

DNase treatment

Enzymes used for plasmid DNA removal were Benzonase nuclease (Novagen, Darmstadt, Germany), and pharmaceutical-grade Pulmozyme (dornase alpha; Genentech, San Francisco, CA). DNase treatment was tested at two time points (Fig. 1). In protocol A DNase was added to the final product after vector harvest and clarification. In protocol B, DNase was added at the end of an 18-hr transfection and before the final medium change. Vector was incubated at 37°C with 5% CO₂ during the DNase treatments. The incubation times and DNase concentrations for both protocols are described in Results.

Residual DNA purification

DNA was extracted by phenol-chloroform purification followed by ethanol precipitation in order to completely deactivate any residual DNase. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1; Sigma-Aldrich, St. Louis, MO) was added to the viral supernatant. The mixture was then vortexed and spun for 10 min at 13,000 rpm. The aqueous phase was removed and an equal volume of chloroform-isoamyl alcohol (24:1; Sigma-Aldrich) was added. The tube was briefly vortexed and spun for 10 min at 13,000 rpm. The aqueous phase was

FIG. 1. Protocols for DNase treatment during lentiviral vector production. (A) In protocol A, DNase is added to harvested vector at the end of production. (B) In protocol B, DNase is added at the end of the transfection period and before medium change and subsequent harvest.

removed and adjusted to 0.3 M sodium acetate. Twice the volume of ice-cold 100% ethanol was added and incubated at -80°C for 2 hr followed by centrifugation at 4°C for 30 min. The pellet was washed with 70% ethanol, centrifuged at 4°C for 30 min, air dried, and then resuspended in water at 65°C for 1 hr.

Real-time PCR

Quantitative polymerase chain reaction (qPCR) was performed with a 7500 real-time PCR system and analyzed with its 7500 system SDS software (Applied Biosystems/Life Technologies, Carlsbad, CA). qPCR was performed with forward and reverse primers (VSVG-F1, 5'-tgcaaggaaagcattgaacaa-3'; VSVG-R1, 5'-gaggagtcacctggacaatcact-3') and a probe [TP-VSVG, 5'(6)-FAM-aggaaactggctgaatccaggttc-TAMRA-3'] specific for a 120-bp fragment of the VSV-G envelope glycoprotein sequence. Isolated DNA was normalized on the basis of the volume of the supernatant tested. Two microliters of the normalized plasmid DNA was added to a PCR master mix consisting of 1 $\times$ TaqMan buffer A, 0.4 mM dNTPs, 1 mM MgCl_2 , 1.2 μM VSVG-F1 and VSVG-R1 primers, 0.4 μM TP-VSVG probe, and AmpliTaq Gold (0.025 U/ μl). Reactions were performed in duplicate or triplicate, using one cycle of 50°C for 2 min, 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 1 min. A standard curve was prepared on the basis of serial dilutions of a VSV-G standard ranging from 10^1 to 10^5 copies.

Measurement of infectious titer

Determination of infectious titer was performed as previously described (Sastry *et al.*, 2002). Briefly, HEK-293 cells were transduced in duplicate, using serial dilutions of vector (1:100 and 1:1000), and titer was based on GFP expression assessed approximately 72 hr later by flow cytometry.

Analyzing plasmid DNA degradation in serum

DNA for the RD114 envelope plasmid was used to analyze the effects of serum and calcium phosphate on DNA

FIG. 2. Plasmid DNA degradation after 1 hr of DNase treatment postharvest. Average qPCR results from four experiments using primers for the VSV-G plasmid as a measure of residual plasmid DNA after DNase treatments. The x axis values represent the units per milliliter of DNase for Pulmozyme (P), Benzonase (B), and an untreated control (Un). Values on the y axis represent the number of gene copies per 500 μl of viral supernatant. Error bars indicate the standard deviation (SD) of the mean.

degradation. Plasmid (50 ng/ μl) was incubated at 37°C with 5% CO_2 in either D10 or serum-free medium. Various incubation times up to 4 hr were observed. After incubation, 5 mM EDTA was added to inhibit any DNase activity and the results were compared by running the products on an agarose gel.

Results

The ability of Pulmozyme to decrease plasmid DNA in lentiviral supernatant was assessed by generating third-generation lentiviral vectors, using a four-plasmid system, including plasmids expressing Gag/Pol, Rev, the VSV-G envelope, and a transgene plasmid expressing GFP. Vector was generated by calcium phosphate transfection, and DNase treatment of the final product was compared with

FIG. 3. Plasmid DNA degradation after 1 hr of DNase treatment performed at the end of transfection. **(A)** Average qPCR results for duplicate experiments using primers for the VSV-G plasmid as a measure of residual plasmid DNA in the final product. DNase treatment was performed at the end of transfection. The x axis values represent the units per milliliter of DNase for Pulmozyme (P), Benzonase (B), and an untreated control (Un). Values on the y axis represent the number of gene copies per 500 μl of viral supernatant. **(B)** Infectious titer of final vector product as assessed by GFP expression, given as infectious units per milliliter. Error bars represent the SD of the mean.

DNase treatment at the end of the transfection (Fig. 1). Plasmid removal was assessed by qPCR with primers specific for the VSV-G envelope gene (the VSV-G plasmid represents approximately one-eighth the total transfected DNA). As a control, Benzonase at 50 U/ml was used, as this concentration of DNase has previously been shown to efficiently remove plasmid DNA from lentiviral products (Sastry *et al.*, 2004).

DNA degradation after treatment with Pulmozyme

The degradation of plasmid DNA after vector harvest (protocol A, Fig. 1) was evaluated with a variety of Pulmozyme concentrations ranging from 1 to 50 U/ml with an incubation time of 1 hr. As shown in Fig. 2, concentrations of Pulmozyme at or above 20 U/ml decreased plasmid DNA to within the limits of detection, and plasmid DNA removal by Pulmozyme was equal to or better than the removal obtained with Benzonase. Increasing the incubation time to 2 or 4 hr did not improve DNA degradation at lower concentrations (data not shown).

Next, the efficiency of DNA degradation was measured when DNase was added at the end of the transfection period and before the final medium change (protocol B, Fig. 1). As

shown in Fig. 3A, Pulmozyme at a concentration of 25 U/ml provided a greater than 2-log decrease in plasmid DNA and was comparable to or better than Benzonase at 50 U/ml. Increasing the Pulmozyme concentration above 25 U/ml did not provide any added benefit (Fig. 3A). Concentrations less than 25 U/ml did not attain a 2-log reduction (data not shown). Any potential adverse effect of Pulmozyme on vector titer was also assessed. As shown in Fig. 3B, the vector titer was not significantly affected by Pulmozyme, even at concentrations up to 100 U/ml.

To determine whether longer incubation times following protocol B would improve the effectiveness of Pulmozyme, the incubation time at the end of the transfection period was increased. As shown in Fig. 4A, addition of Pulmozyme at 25 U/ml led a similar amount of residual DNA in the final product after 1, 2, and 4 hr of incubation. To evaluate whether longer incubations allowed for a decrease in Pulmozyme concentrations, a 4-hr incubation was performed with concentrations varying from 5 to 25 U/ml. As shown in Fig. 4B, the lowest DNA content was noted at 20 and 25 U/ml. The 4-hr incubation did not alter the vector titer (Fig. 4C). When taking the data in aggregate, adding Pulmozyme at 20 U/ml after transfection and incubating for 1 hr gave optimal DNA degradation, and results were similar to or better than with

FIG. 4. Plasmid DNA degradation over time when treating at the end of transfection. **(A)** qPCR results for VSV-G plasmid in the final product when Pulmozyme (25 U/ml) is added at the end of transfection for 1–4 hr. Values on the y axis represent the number of gene copies per 500 μ l of viral supernatant. **(B)** qPCR results on residual VSV-G after a 4-hr incubation with various concentrations of DNase. The x axis values represent the units per milliliter of DNase for Pulmozyme (P), Benzonase (B), and an untreated control (Un). Values on the y axis represent the number of gene copies per 500 μ l of viral supernatant. **(C)** Infectious titer of final vector product as assessed by GFP expression, given as infectious units per milliliter. Error bars represent the SD of the mean.

Benzonase at 50 U/ml. Unfortunately, the level of DNA degradation when treating after transfection (protocol B) was significantly less than that obtained by treating after vector harvest (protocol A).

DNA degradation in the presence of serum

A significant difference between the vector treated in protocol A versus protocol B is the presence of serum in the medium. Specifically, in protocol A the vector is treated in serum-free medium, whereas the vector in protocol B is treated in serum-containing medium. To evaluate the effectiveness of DNase in the different media we incubated plasmid DNA in serum-free medium (OptiPRO), and in D10 medium (DMEM with 10% fetal bovine serum), for up to 4 hr (37°C, 5% CO₂). Interestingly, free plasmid DNA is stable in serum-free medium (Fig. 5A), whereas plasmid in medium containing fetal bovine serum leads to a time-dependent degradation of plasmid DNA (Fig. 5B). Complexing plasmid DNA with calcium phosphate provides some protection from serum degradation (Fig. 5C). This is consistent with observations that calcium phosphate can form complexes with DNA that are resistant to extracellular DNase activity (Loyter *et al.*, 1982). However, complexing plasmid DNA with calcium phosphate does not appear to provide the same degree of protection in the presence of Pulmozyme (Fig. 5D).

These findings suggest that the lower DNA degradation seen with protocol B is not the result of Pulmozyme inhibition by serum.

Cost analysis

Another consideration when assessing the feasibility of replacing Benzonase with Pulmozyme is the cost. Currently, the cost of Benzonase is \$6.60 per 1000 units compared to \$26.80 for 1000 units of Pulmozyme. For treating 20 liters of unconcentrated product, the cost of Benzonase (50 U/ml) is estimated at \$6600 while the cost of Pulmozyme (20 U/ml) is estimated at \$10,720. To decrease costs, we currently concentrate vector product 10-fold before DNase treatment. Treating 2 liters of product decreases the cost for Pulmozyme treatment to \$1,072 adding \$412 to the cost of vector production when compared to the use of Benzonase. With the supply cost of a 20-liter clinical-grade vector production estimated at approximately \$25,000, the switch to Pulmozyme would increase the cost by approximately 1.6%.

Discussion

The studies presented here demonstrate that Pulmozyme can be used as an alternative to Benzonase for removing residual plasmid DNA from lentiviral vector products.

FIG. 5. The effects of serum and calcium phosphate on plasmid DNA degradation. Plasmid incubated in (A) serum-free medium (OptiPRO) or (B) D10 (serum-containing) medium; (C) plasmid complexed with calcium phosphate incubated in D10 medium; and (D) plasmid complexed with calcium phosphate incubated in D10 medium with Pulmozyme (25 U/ml). Each sample began with RD114 plasmid at 50 ng/ μ l and was analyzed on a 1.2% agarose Tris-acetate-EDTA (TAE) gel.

Pulmozyme offers the advantage of being a human protein produced in Chinese hamster ovary (CHO) cells as opposed to the bacterially derived Benzonase. Most importantly, the availability of a pharmaceutical grade reagent will be important as vectors move from investigational agents into licensed products and the requirements for product purity escalate.

Incorporating the DNase treatment into the transfection process was evaluated as a means to decrease the time between product harvest and freeze down. While lentiviral vector half-life is longer than that of traditional gamma-retroviral vectors, efforts to insure a rapid freezing will help maximize the final vector titer (Higashikawa and Chang, 2001) and treatment at the end of transfection (protocol B) would minimize the time between vector harvest and freezing. The other advantage of DNase treatment in the transfection process is that subsequent media changes will greatly decrease the concentration of DNase in the final product. In contrast, treating the harvested vector leaves significant amounts of DNase in the final product (Zufferey, 2002) and exposing patients to foreign proteins has been associated with adverse events. For example, patients with pneumonia, treated with a bovine DNase, developed severe respiratory reactions due to contaminating proteins (Johnson *et al.*, 1954; Lachmann, 1967; Raskin, 1968). Unfortunately, treatment at the end of transfection was inferior to treating the harvested product in terms of DNA degradation.

The cause of the disparity observed between the two protocols is not readily apparent. Our findings suggest that although there does not appear to be any inhibition of the DNase by serum, there is still some degree of protection when complexed with calcium phosphate. This is apparent since Pulmozyme is able to degrade plasmid DNA complexed with calcium phosphate (Fig. 5D), yet there are consistent levels of plasmid DNA detected by qPCR (Figs. 3 and 4). This indicates that plasmid DNA complexed with calcium phosphate infers some resistance to DNase degradation, and is consistent with previously reported findings (Loyter *et al.*, 1982). The ability to degrade the residual DNA more efficiently by treating the harvested vector is likely due to the dynamic and relatively unstable nature of the calcium phosphate complex over time (Jordan and Wurm, 2004).

Although our findings indicate Pulmozyme is a viable DNase, the timing and concentration will likely need to be tailored to the vector production method employed. At present, there is considerable variability in large-scale lentiviral production methods, ranging from methods that provide minimal processing to those with extensive diafiltration, ion-exchange, and size separation (Slepushkin *et al.*, 2003; Transfiguracion *et al.*, 2003; Merten *et al.*, 2011). Purification steps are likely to reduce DNA content, as well as decrease the amount of residual Pulmozyme in the final product. It is possible that lower concentrations of Pulmozyme could be used if subsequent purification steps contributed to plasmid DNA removal so that combined the level of DNA is decreased to undetectable levels. For clinical products, residual foreign proteins must also be minimized in order to avoid allergic reactions after vector administration. At present, the U.S. FDA has not set limits on the amount of residual DNase in a final product. The acceptable level may vary depending

on whether the product is administered *ex vivo* or *in vivo*. Therefore Pulmozyme removal should be considered in the design of downstream processing, with highly stringent processes used for vector injected directly into immune-competent individuals.

The variability in production methods, along with evolving technology suggest that ongoing refinements make universal statements about DNase treatment difficult. Nevertheless, the ability to substitute Benzonase with an FDA-approved reagent will improve the safety of vector products without compromising vector titer. This change will result in a minimal increase in the overall cost of clinical vector products.

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Author Disclosure Statement

Kenneth Cornetta is a founder of Rimedion, but there is no financial interest in the work described in this manuscript.

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