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Nebulization of single-chain tissue-type and single-chain urokinase plasminogen activator for treatment of inhalational smoke-induced acute lung injury

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A B S T R A C T

Single-chain tissue-type plasminogen activator (sctPA) and single-chain urokinase plasminogen activator (scuPA) have attracted interest as enzymes for the treatment of inhalational smoke-induced acute lung injury (ISALI). In this study, the pulmonary delivery of commercial human sctPA and lyophilized scuPA and their reconstituted solution forms were demonstrated using vibrating mesh nebulizers (Aeroneb® Pro (active) and EZ Breathe® (passive)). Both the Aeroneb® Pro and EZ Breathe® vibrating mesh nebulizers produced atomized droplets of protein solution of similar size of less than about 5 µm, which is appropriate for pulmonary delivery. Enzymatic activities of scuPA and of sctPA were determined after nebulization and both remained stable (88.0% and 93.9%). Additionally, the enzymatic activities of sctPA and tcuPA were not significantly affected by excipients, lyophilization or reconstitution conditions. The results of these studies support further development of inhaled formulations of fibrinolysins for delivery to the lungs following smoke-induced acute pulmonary injury.

1. Introduction

Tissue-type plasminogen activator (tPA) and urokinase plasminogen activator (uPA) are fibrinolysins, which are proteins that generate plasmin, which in turn degrade fibrin. When given by intravenous administration, these agents have been used for the treatment of various types of thrombotic disorders such as submassive/massive pulmonary embolism [1], myocardial infarction [2], ischemic stroke [3–5] and deep vein thrombosis [6,7]. These same agents have also been administered at extravascular sites of inflammation associated with florid transitional fibrin deposition. Intrapleural administration of fibrinolysins has been used to expedite the drainage of loculated pleural effusions, including those associated with empyema or complicated parapneumonic pleural effusions [8–10]. It has also been demonstrated that airway fibrin in a murine model of asthma caused bronchospasm that was relieved by airway administration of tPA [11]. Prior reports further suggest that administration of fibrinolysins to the airways and lung parenchyma may be of benefit in acute respiratory distress syndrome (ARDS) [12–15]. ARDS, often progressing from a severe acute lung injury (ALI), can be caused by sepsis, aspiration or by inhalational exposures and carries a high mortality rate (30–40%) as well as occasional long-term morbidity [16,17].

Inhalational smoke-induced ALI (ISALI) is characterized by severe airway obstruction and fibrinous cast formation in the airways. tPA has been reported to effectively improve gas exchange and other physiologic outcomes when administered by repeat dose nebulization in a sheep model of ISALI [18]. Previous studies have also reported that human tPA suppresses activator-induced neutrophil superoxide anion production and has an anti-inflammatory effect on ARDS in a preclinical rodent model after pulmonary administration of tPA [19,20]. In a model of sulfur mustard exposure in rats, tPA also improved outcomes of ALI [21]. These studies collectively provide proof of concept that tPA can exert salutary effects in models of ALI. An effective route of tPA delivery for treatment of ARDS is direct administration to the airways

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[22], which limits potential systemic toxicity. However, vulnerability for airway bleeding is a potentially serious complication of this mode of administration [23]. These considerations justify the current scope of work designed to enable testing of preparations of fibrinolysins to more safely and effectively treat ISALI and related forms of airway diseases associated with prominent fibrin deposition [18,21].

uPA may likewise be potentially useful for treatment of ARDS. Nebulization of the two chain form of uPA decreased lung fibrosis in rabbits with bleomycin (BLM)-induced lung injury, which is characterized by early ALI and accelerated pulmonary fibrosis [24]. This observation is consistent with the findings observed in transgenic and knockout mice. Both the up-regulation of uPA and the down-regulation of it's major inhibitor; plasminogen activator inhibitor-1 (PAI-1) attenuated accelerated pulmonary fibrosis induced by BLM [25]. These findings and the high levels of PAI-1 found in the bronchoalveolar lavage fluids of patients with ARDS and primates with ALI also characterized by robust extravascular fibrin deposition in the lungs [26,27], suggest that administration of a nebulized PAI-1-resistant fibrinolysin could potentially be of therapeutic advantage in ALI. The proenzyme form of uPA, single-chain uPA (scuPA), is relatively resistant to inhibition by PAI-1 [28]. A preliminary study, as well as other literature, strongly suggests that scuPA is advantageous for ISALI and was hypothesized that scuPA should be more effective and less prone to airway bleeding than tPA [29,30]. It was reported that scuPA generates durable plasminogen activator activity and that it is safe and effective in clearing intrapleural fibrin in rabbits [28,31,32].

Local treatment of ISALI can involve pulmonary delivery of the enzyme directly to the lungs, which allows for administration of lower doses than needed for systemic delivery and theoretically mitigates against systemic side effects [33]. Nebulization is one of the techniques for airway and pulmonary delivery of protein and peptide drugs in solution or suspension forms. However, after only a few minutes of nebulization, proteins can denature to reduce their activity, form protein aggregates or increase immunogenic potential [34]. These effects may result from excessive stress on the protein residing within the droplets and the bulk reservoir of the nebulizer. Therefore, experimental confirmation of a nebulization paradigm is a significant step in the development of pulmonary delivery systems for interventional agents including proteins.

Although a large number of biologicals have successfully been used therapeutically, Pulmozyme® remains the only commercially available enzyme that is delivered by inhalation [34]. To our knowledge, there are no commercially approved inhaled products containing plasminogen activators. Major challenges of formulating plasminogen activators include sufficiency of the aerosol generation, appropriate deposition and retention of activity [34]. Most of these proteins are not stable in liquid form [35] and are more stable in solid form [36]. Hence, lyophilization can prolong the stability of the protein in order to increase shelf-life, which is necessary for commercialization [37–39].

The feasibility of single-chain tissue-type plasminogen activator (sctPA) for pulmonary delivery was reported in Dunn's study [22]. The sctPA formulations containing different concentration of polysorbate 80 in the range of 0.05–0.1%w/w were optimized and the stability of sctPA was evaluated after nebulization. It was found that the enzyme activity of sctPA did not decrease after nebulization using a jet nebulizer [22]. Polysorbate 80 functions as a nonionic surfactant and may enhance the stability of protein during nebulization [40–42]. However, the functions of the excipients can vary by type, grade and concentration. Therefore, the stability of sctPA in the commercial human sctPA (Cathflo® Activase®), which contains only 0.002% w/w of polysorbate 80, was evaluated during nebulization.

Munster et al. reported the feasibility of scuPA as a nebulized formulation using an air-jet and ultrasonic nebulizer, but did not study vibrating mesh nebulizers (VMNs). The scuPA solutions were nebulized using a Ventstream air-jet nebulizer or a Syst's AM DP-100 ultrasonic nebulizer. Aerosol characteristics and drug output were reported. The effect of nebulization on the enzyme activity of scuPA was also reported. It was shown that nebulization using an air-jet nebulizer did not affect the enzyme activity of scuPA, while an ultrasonic nebulizer caused partial inactivation of scuPA. Although droplets generated from both nebulizer types were in the respirable size range, the drug output from both nebulizers was only 50% [43]. To minimize drug wastage, VMNs were chosen in the present study.

VMNs reportedly have high output efficiency [44,45]. Additionally, VMNs are recommended to aerosolize protein and peptide formulations to mitigate against protein degradation and inactivation which have been reported for other devices [34,46,47]. Overall, the impact of nebulization on stability of protein depends on the characteristics of the individual protein [34]. Some proteins such as granulocyte-colony stimulating factor, lactate dehydrogenase or immunoglobulin G type 1 antibody lose their activity during nebulization using VMNs [48]. Furthermore, reports of the effect of heating within the medication reservoir during nebulization using VMNs are inconsistent. Some studies reported that heating of the drug reservoir does not occur during nebulization using VMNs [34,49,50]. In contrast, a significant increase in the temperature within the drug reservoir was found during nebulization using the PARI eFlow® and Aeroneb® Go nebulizers, which may cause aggregation of thermolabile proteins [34]. Therefore, another objective of this study was to determine if reconstituted solutions of sctPA and scuPA maintain their enzyme activity following nebulization using a VMN.

2. Materials and methods

2.1. Materials

Human recombinant sctPA (Cathflo® Activase® was purchased form Genentech, Inc., South San Francisco, CA). scuPA (Specific activity of bulk solution of 124,000 IU/ml of bulk solution) was manufactured by NIH SMARTT under contract # HHSN268201100014C (S. Idell, PI). D-mannitol was purchased from Thermo Fisher Scientific Inc. (Bridgewater, NJ), and Dulbecco's phosphate buffered saline (DPBS) was purchased from Lonza (Walkerville, MD). BSA was purchased from Sigma-Aldrich (St. Louis, MO) and human plasmin was purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Urokinase (high molecular weight two chain form, 100,000 IU/mg) was obtained from Sekisui Diagnostics (Lexington, MA), and Pefachrome uPA (Bz-β-Ala-Gly-Arg-pNA-AcOH) and Pefachrome tPA were purchased from Pentapharm (Switzerland). NuPAGE® Novex® Bis-Tris Midi Gels (Life Technologies, Carlsbad, CA) and 96-well plates were purchased from Thermo Fisher Scientific Inc. (Bridgewater, NJ). Pierce™ BCA protein assay kit was purchased from Thermo Fisher Scientific Inc. (Bridgewater, NJ). All chemical reagents and solvents used were at least analytical grade.

2.2. Freeze-thaw cycle stability of scuPA

Frozen scuPA in citrate buffer was thawed to 25 °C and diluted with DPBS to 0.25 mg/ml. The scuPA solutions were frozen at −80 °C for 1 h and then thawed at 25 °C for 1 h. This freeze-thaw cycle was repeated for three cycles. The samples were collected after each cycle and kept at −20 °C until analyzed.

2.3. Solid-state preparation of scuPA

Lyophilization was utilized to prepare freeze-dried scuPA. In brief, a bulk solution of scuPA in DPBS containing 500 μg/mL of scuPA with 1.5% w/v of mannitol was prepared and sterilized by filtration through 0.2 μm surfactant-free cellulose acetate sterile syringe filter (Corning Inc., Corning, NY). Two mL of the filtrate was placed in a borosilicate glass vial and lyophilized it using a VirTis Advantage Lyophilizer (VirTis Company Inc., Gardiner, NY). Lyophilization cycle parameters

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