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Peptide Amphiphile Nanofiber Delivery of Sonic Hedgehog Protein to Reduce Smooth Muscle Apoptosis in the Penis after Cavernous Nerve Resection

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Abstract

Introduction—Erectile dysfunction (ED) is a serious medical condition that affects 16–82% of prostate cancer patients treated by radical prostatectomy and current treatments are ineffective in 50–60% of prostatectomy patients. The reduced efficacy of treatments makes novel therapeutic approaches to treat ED essential. The secreted protein Sonic hedgehog (SHH) is a critical regulator

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of penile smooth muscle and apoptosis that is decreased in cavernous nerve (CN) injury and diabetic ED models. Past studies using Affi-Gel beads have shown SHH protein to be effective in suppressing apoptosis caused by CN injury.

Aim—We hypothesize that SHH protein delivered via novel peptide amphiphile (PA) nanofibers will be effective in suppressing CN injury-induced apoptosis.

Methods—Adult Sprague Dawley rats (n = 50) were used to optimize PA injection in vivo. PA with SHH protein (n = 16) or bovine serum albumin (BSA) (control, n = 14) was injected into adult rats that underwent bilateral CN cut. Rats were sacrificed at 2, 4, and 7 days. Alexa Fluor-labeled SHH protein was used to determine the target of SHH signaling (n = 3).

Main Outcome Measures—Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) and semi-quantitative immunohistochemical analysis for SHH protein and cluster differentiation protein three (CD3) were performed.

Results—SHH-PA caused a 25% and 16% reduction in apoptosis at 4 and 7 days after CN injury and a 9.3% and 19% increase in SHH protein at 4 and 7 days after CN injury. CD3 protein was not observed in SHH-PA-treated penis. In vitro, 73% of SHH protein diffused from PA within 6 days. Labeled SHH was observed in smooth muscle.

Conclusions—PA technology is effective in delivering SHH protein to the penis and SHH is effective in suppressing CN injury-induced apoptosis. These results suggest substantial translational potential of this methodology and show that only a short duration of SHH treatment is required to impact the apoptotic index.

Keywords

Penile Smooth Muscle; Sonic Hedgehog; Peptide Amphiphile; Apoptosis; Nanotechnology; Cavernous Nerve Injury

Introduction

Researchers focusing on in vivo drug delivery are continually searching for novel materials and strategies to match the unique environment of the human body. A multitude of various materials (small molecules, polymers, inorganic nanoparticles) can be processed into a range of delivery modalities (liposomes, gels, composite charged assemblies) to deliver their desired cargo (small molecules, DNA, proteins) to a desired tissue of interest. Supramolecular self-assembly has demonstrated significant promise in this field, since the individual substrate molecules can be preprogrammed to arrange into an ordered structure on the nanoscale or microscale, matching the size requirements for many cargo and delivery needs. Many self-assembly strategies yield micellar or vesicular objects in aqueous suspension, which can be easily injected to a site of interest. The present study employs a unique family of materials called peptide amphiphiles (PA), which self-assemble into high-aspect-ratio nanofibers. At sufficient concentrations, these nanofibers then entangle to create macroscopic hydrogels. The advantage to this methodology for protein delivery in vivo is that the unassembled PAs can be injected in solution with the protein to a site of interest, and quickly triggered to assemble into a stable hydrogel, with complementary physical properties to the surrounding soft tissue [1–4]. Additionally, PA nanofibers are noninvasive, biodegradable, and elicit no significant immune response.

Classical PAs consist of a linear hydrophobic tail coupled to a peptide block that includes β -sheet-forming segments, charged residues for solubility, and optional biological epitopes [5,6] (Figure 1A). Upon application of a trigger, such as a change in pH or ion concentration, these PA molecules self-assemble in aqueous solution into nanofibers and

form a gel with extended release properties [5,6]. The protein structure is protected within the gel matrix, and no chemical modification of the protein is necessary [1,7,8]. While recently developed, this type of nano-scaffold has been used successfully in vivo to deliver vascular endothelial growth factor (VEGF) in other tissues for angiogenesis induction [1,7], and to maintain high local transforming growth factor beta (TGF- β 1) concentrations for mesenchymal stem cell differentiation [3,4]. In this study, we propose to utilize PA methodology to deliver a previously identified apoptosis suppressant to the penis to prevent the development of erectile dysfunction (ED) in a prostatectomy rat model. PA technology is particularly useful for the penis because the PA molecules, which are a liquid, can be combined with the protein of interest and CaCl₂ (ionic trigger) and injected directly into the sinuses of the corpora cavernosa, where within a short time (minutes), the gel scaffold will form, coating the sinusoidal lining for extended release of protein over several days. While beyond the scope of this study, PA technology can be developed for translational application in humans at the time of prostatectomy by substituting human protein for rodent. PA technology can also be used as a delivery vehicle for any protein to the penis, making it a powerful, biodegradable tool for extended release of proteins in vivo.

The reason that in vivo protein delivery to the penis is required is because of ED, a significant medical concern that affects approximately half of adult males between the ages of 40 and 70 years. Underlying conditions that contribute to the development of ED are age, coronary artery disease, peripheral vascular disease, smoking, dyslipidemia, diabetes, and radical prostatectomy [9]. Also, 16–82% of prostate cancer patients treated by radical prostatectomy experience ED [10] and phosphodiesterase type 5 inhibitors are ineffective in 50–60% of prostatectomy patients who experience ED [11], depending on their nerve injury status. The reduced efficacy of treatments in this population makes novel therapeutic approaches to treat ED essential. Significantly increased apoptosis of penile smooth muscle is common in animal models with ED and is a critical factor in whether ED occurs [12–14]. We propose that abundant apoptosis observed in penile smooth muscle when the cavernous nerve (CN) is injured (mimicking neural injury caused by prostatectomy) is a major contributing factor to ED development. If penile apoptosis could be prevented following prostatectomy while the CN regenerates, then resumption of normal erectile function would occur more quickly, and irreversible morphological changes in the penis that cause ED would be prevented. Understanding the mechanisms that regulate smooth muscle apoptosis in the penis is critical for development of new therapeutic approaches for ED treatment and prevention.

The protein that we would like to utilize PA methodology to deliver to the corpora cavernosa of the penis is the secreted glycoprotein Sonic hedgehog (SHH), which is an essential regulator of penile smooth muscle and apoptosis that is critical for normal erectile function [14–17]. SHH is synthesized as a 45–49 kDa secreted precursor that undergoes autoproteolytic cleavage to yield two mature proteins: a 19-kDa amino-terminal fragment which is cholesterol modified, palmitoylated, and is biologically active, and a 25–31 kDa carboxy-terminal fragment that retains no known biological activity [18–20]. SHH is necessary during embryogenesis of the penis for both genital tubercle outgrowth and differentiation [21] and in mice with a targeted deletion of *Shh*, external genitalia were completely absent [22]. The SHH pathway functions after birth to direct differentiation of corpora cavernosal sinuses [15] and in the adult penis SHH functions to maintain the sinusoid morphology of the corpora cavernosa [15] that it helped establish. When SHH signaling is inhibited in the adult penis, there is a significant 12-fold increase in smooth muscle apoptosis [14], which alters sinusoidal morphology and which causes ED [15]. In previous studies, it has been shown that SHH protein is decreased in the penis of two commonly studied rat models of ED, the BioBreeding/Worcester (BB/WOR) diabetic rat [16] and the CN-injured Sprague Dawley rat [14]. We have recently shown the efficacy of

apoptosis suppression in rats when SHH protein is delivered locally to the penis at the time of CN injury [14], indicating that SHH has significant potential to be developed as a treatment to prevent ED by suppressing post prostatectomy apoptosis. The Affi-Gel bead technology used in these studies is not applicable to humans, because it is not biodegradable, and the beads are not delivered uniformly or throughout the entire penis. We propose to use a novel self-assembling nanofiber vehicle (PA) for SHH protein delivery to the penis to suppress CN injury-induced apoptosis. This methodology is locally administered and can be adapted for translational application to humans post prostatectomy. Increasing SHH protein abundance via shRNA or viral gene delivery is unlikely to be effective since there is a blockade in SHH protein synthesis when the CN is injured [14]. This application of SHH, to suppress CN injury-induced apoptosis and preserve corpora cavernosal morphology while the CN regenerates, is innovative and has significant clinical relevance.

In this study, we hypothesize that SHH protein delivered via biodegradable nanofiber PAs will be effective in suppressing apoptosis induction caused by CN injury and can be developed into a therapy to prevent post prostatectomy apoptosis in humans. We have examined this hypothesis by synthesizing PAs for SHH protein delivery to the penis, by optimizing PA gelation, injection, and stability, and by examining the effectiveness of SHH-PA delivery in vivo for apoptosis suppression in a Sprague Dawley rat CN injury model.

Materials and Methods

Animals

Sprague Dawley rats postnatal day 115–120 (P115–P120) were obtained from Charles River. All animals were cared for in accordance with Institutional Animal Care and Use Committee (IACUC) approval and the National Research Council publication *Guide for Care and Use of Laboratory Animals*.

PA Synthesis

PAs were synthesized at the Northwestern Institute for BioNanotechnology in Medicine Chemistry Core Facility. PAs were synthesized by standard methods via Fmoc-based solid phase synthesis on Wang resins, preloaded with a glutamic acid residue [23]. Through standard deprotection and coupling procedures, the VVVA AEEEE sequence was synthesized on the resin beads and the batch was split. One batch was terminated in a palmitic acid (C16) alkyl tail. The other portion was coupled first to Fmoc-6-aminohexanoic acid, deprotected, and coupled to 1-pyrenebutyric acid. Both batches were cleaved from the resin, precipitated in cold ethyl ether, and lyophilized to a powder. PAs were purified by preparative high performance liquid chromatography (HPLC) and lyophilized to a powder. Each dry powder was redissolved in water, adjusted to pH 7 using dilute sodium hydroxide, re-lyophilized, and stored at -80°C as a dry powder until needed. The final PAs are shown in Figure 1A as (C16)-V₃A₃E₃-COOH (molecular weight = 1,154 g/mol) and pyrene-V₃A₃E₃-COOH (molecular weight = 1,301 g/mol). Compositions were confirmed by mass spectrometry. Pyrene was added to PA as a fluorescent marker.

Optimization of PA Injection

Adult Sprague Dawley rats were used to optimize PA injection methodology, including volume of PA injected, whether a silk tie was required to localize the PA within the penis while it formed the gel in vivo, where along the length of the penis, the injection of PA should occur in order to maximize dispersion throughout the entire corpora cavernosa and duration of PA retention in vivo. The pyrene-functionalized fluorescent PA (10 mM final concentration) was mixed briefly in 1:1 (v/v) ratio with a CaCl₂ (20 mM final concentration) solution, drawn into a syringe, and injected directly into the corpora cavernosa. Also, 20, 50,

and 100 μ L of PA were used for this study to optimize PA volume ($n = 5$ at each volume). PA was visualized in the penis under ultraviolet (UV) light after injection. Injections were performed both with ($n = 5$) and without ($n = 5$) a silk tie (single knot) placed around the base of the penis prior to injection with 100 μ L of PA and an equal volume of CaCl_2 . The total time the silk tie was in place was ~ 5 minutes. Injections were made into both the proximal ($n = 5$) and distal ($n = 5$) regions of the penis to determine which injection site yielded better dispersion of the PA throughout the penis. Once optimization of injection methodology took place, the duration of the PA was examined by injecting 50 μ L of PA and an equal volume of CaCl_2 into the penis. Rats were sacrificed after 1 hour ($n = 5$), after 7 days ($n = 5$), and after 14 days ($n = 5$). Since injection was made directly into the sinuses of the corpora cavernosa, which had a tie placed around the penis, the liquid PA and CaCl_2 flowed throughout the sinusoidal spaces in the corpora cavernosa and then gelled in this same region in vivo.

PA Delivery of SHH Protein to CN Cut Sprague Dawley Rats

P120 Sprague Dawley rats were randomized into two groups: bilateral CN resection and SHH PA treatment ($n = 12$) and bilateral CN resection and BSA PA treatment ($n = 11$) which was used as a control group. Bilateral CN resection was performed as described previously [24]. Briefly, a 5-mm section of the CN was removed 5-mm from the pelvic ganglia bilaterally using a KAPS (Asslar, Germany) industrial microscope under direct vision through a midline abdominal incision. A 100-mM solution of PA (50 μ L) was added to 5 μ L of a solution of SHH protein in water (1.25 μ g/ μ L, R&D Systems, Minneapolis, MN, USA). A 200 mM CaCl_2 solution (50 μ L) was added to the SHH-PA solution. The skin covering the penis was then retracted and the PA was immediately injected directly into the corpora cavernosa with a 26-gauge needle (105 μ L volume) where the PA formed a loose gel lining the sinuses within 30 seconds to 1 minute. The final concentration of PA was 10 mM and CaCl_2 was 20 mM. The final amount of SHH protein injected was 6.25 μ g per rat. Penises were harvested from euthanized males by sharp dissection 2, 4, and 7 days after SHH protein/PA/ CaCl_2 injection and were frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Double the amount of SHH protein (12.5 μ g, $n = 4$) or BSA (12.5 μ g, $n = 3$) in the PA carrier was also injected into Sprague Dawley rats which were sacrificed after 4 days.

Immunohistochemical Analysis

Immunohistochemical (IHC) was performed as previously outlined [14–16] on penis tissue assayed with goat polyclonal anti-SHH (1/100, N-19, SC-1194, Santa Cruz) and anti-patched (PTCH1, 1/100, G-19, SC-6149, Santa Cruz), and mouse monoclonal anti-cluster differentiation protein three (CD3, 1/50, SC-52382). The secondary antibodies used were Alexa Fluor 488 chicken antigoat (1/300, Molecular Probes, Carlsbad, CA), and Alexa Fluor 488 chicken anti-mouse (1/200, Molecular Probes). Negative controls were performed with secondary only (without primary) to test for nonspecific staining and auto-fluorescence. Sections were mounted using Pro-Tex Mounting Medium (Baxter Diagnostics, Inc., Pittsburgh, PA, USA). Microscopy was performed using a fluorescent microscope (Leitz) and photographed using a Nikon digital camera. Quantification of SHH protein was performed using the Image J program [25]. Total fluorescence was measured in five fields from each section and five sections for each tissue. For subcellular localization studies of SHH and PTCH1, propidium iodide (0.052 μ g/ml) was used as a stain for the nucleus.

TUNEL Assay for Apoptosis

TUNEL assay was performed using the Apoptag kit (Chemicon International, Temecula, CA, USA) on isolated penis tissue fixed overnight at 4°C in 4% paraformaldehyde, embedded in paraffin and sectioned 16 μ m in thickness as described previously [14]. All

cells were counterstained using 4',6'-diamidino-2-phenylindole (DAPI) (0.005 $\mu\text{g/mL}$). Fluorescent apoptotic cells were observed under a fluorescent microscope (Leitz) and photographed using a Nikon digital camera. Quantification of apoptosis was performed by counting the total number of cells and the number of apoptotic cells in a given field selected at random by visual observation. The number of apoptotic cells/all cells (apoptotic index) in five fields from each section and five sections for each penis were counted.

SHH Protein Dissociation from the PA In Vitro

100 μL of 100 mM PA ((C₁₆)-V₃A₃E₃-COOH) was added to 6.25 μg SHH protein (R&D Systems) in an Eppendorf tube. The mixture was pipetted into a well of a 96 well plate and 100 μL of 200 mM CaCl₂ was added directly to the well. Also, 100 μL PA was used for this study to completely cover the bottom of each well with PA. Gel formation took place within 30 seconds to 1 minute. The gel was allowed to completely set for 10 minutes prior to adding 60 μL of a modified Ringer's solution (100 mL of a solution containing = 600 mg NaCl, 30 mg KCl, and 20 mg CaCl₂ in 1X phosphate buffered solution [PBS]) to the top of the well in order to mimic the in vivo extracellular CaCl₂ concentration in the penis. Four wells were formed that contained SHH and PA and two wells that contained PA only (used as a blank). Also, 50 μL was removed from each well and were replaced with a fresh solution at 1, 3, 5, 7, 21, 29, 46, 53, 70, 95, 122, and 141 hours. Samples were measured at an absorbance of 280 nm in a Beckman Spectrophotometer (DU 640, Fullerton, CA, USA). The absorbance in four wells was averaged for each time point and the cumulative percent release of protein from PA was reported $\pm$ standard error of the mean (SEM).

Fluorescent Labeling of SHH Protein

Fluorescent labeling of SHH protein (R&D Systems) was performed using the Alexa Fluor 488 Microscale Protein Labeling Kit (Molecular Probes) according to manufacturer's instructions. Briefly, 25 μg SHH protein was dissolved in 25 μL water. Also, 2.5 μL of a 1M sodium bicarbonate buffer was added to SHH protein. Furthermore, 2.9 μL of reactive dye was added to the reaction tube and the solution was incubated for 20 minutes at room temperature. The reaction mixture was added to a previously prepared spin column and spun at 14,000 $\times g$ for 1 minute. Labeled SHH protein was collected and the total protein and fluorescence were quantified by measuring the absorbance at 280 and 494 nm on a Beckman Spectrophotometer (DU 640).

Fluorescent-Labeled SHH Protein Injection into Penis

P120 Sprague Dawley rats underwent bilateral CN resection and SHH PA treatment (n = 4) as outlined above. Briefly, a 5-mm section of the CN was removed 5 mm from the pelvic ganglia bilaterally using a KAPS Industrial microscope under direct vision through a midline abdominal incision. A 100 mM solution of PA (50 μL) was added to 23 μL of a solution of Alexa Fluor 488-labeled SHH protein in water (0.24 $\mu\text{g}/\mu\text{L}$, R&D Systems). A 200-mM CaCl₂ solution (50 μL) was added to the SHH-PA solution. The skin covering the penis was then retracted and the PA was immediately injected directly into the corpora cavernosa with a 26-gauge needle (123 μL volume) where the PA formed a loose gel lining the sinuses within 30 seconds to 1 minute (n = 3). The final amount of SHH protein injected was 5.4 μg per rat. Penes were harvested from euthanized males by sharp dissection 3 hours after SHH protein/PA/CaCl₂ injection and were frozen in liquid nitrogen prior to cutting 16 μM frozen sections and examining the localization of labeled protein using a fluorescent microscope.

Statistics

Statistics were performed using the Excel program (Microsoft) and the results are reported $\pm$ the SEM. A *t*-test was performed to determine significant differences in protein abundance. Differences ≤ 0.05 are considered significant.

Results

Optimization of SHH PA Injection and Duration

Pyrene-functionalized PA (which is fluorescent) was used for optimization studies so that the PA location within the penis could be tracked by observation of fluorescence. In each trial, equal volumes of pyrene-PA and CaCl_2 solution were mixed and injected, to yield a final gelled volume. In addition, 20, 50, and 100 μL of PA and an equal volume of CaCl_2 were injected into adult Sprague Dawley rats in order to optimize PA volume ($n = 5$ at each volume). When 20 μL fluorescent PA was injected into the corpora cavernosa of the penis, PA was visualized under UV light as a highly brightened region of the penis (Figure 1B). When 50 μL of PA and an equal volume of CaCl_2 were injected into the penis, the entire penis had visible fluorescent PA (Figure 1C). The optimum injection volume was found to be 50 μL PA and 50 μL CaCl_2 (total volume of 100 μL). With this volume of PA, a gel forms throughout the entire penis uniformly and was visible under dark light as a bright glow in the entire corpora cavernosa (Figure 1C). When less PA was injected, the PA did not fill the entire corpora cavernosa (Figure 1B). When 100 μL of PA and an equal volume of CaCl_2 were injected into the penis, the PA did not remain localized within the penis (data not shown).

Injections were performed both with ($n = 5$) and without ($n = 5$) a silk tie (single knot) placed around the base of the penis prior to injection with 50 μL of PA and an equal volume of CaCl_2 . The total time the silk tie was in place was ~ 5 minutes. It was found that PA gelled better and remained localized completely in the penis when a silk tie was used.

Injections were made into both the proximal ($n = 5$) and distal ($n = 5$) regions of the penis to determine which injection site yielded better dispersion of the PA throughout the penis. When PA was injected into the distal portion of the penis, fluorescence was observed well dispersed throughout both corpora cavernosa (Figure 1C). However, when PA was injected into the proximal portion of the penis, PA did not appear to reach all of the adjacent corpus cavernosum (data not shown).

One hour after PA was injected into the penis, histological sectioning revealed the presence of PA within the corpora cavernosa, forming a thin layer lining the sinusoidal spaces (Figure 1D, $n = 5$). A normal penis without PA is shown for comparison (Figure 1D). PA was still abundant and clearly visible after 7 days within the corpora cavernosa ($n = 5$), despite the presence of shear stress caused by blood flow within the tissue (Figure 1E). By 14 days, PA was observed as a nonspecific fluorescence within the corpora cavernosa indicating that the PA was mostly degraded by 14 days after injection (Figure 1F, $n = 5$).

SHH Protein Quantification in SHH-PA and BSA-PA-Treated Corpora Cavernosa

SHH protein or BSA (control) were delivered to corpora cavernosa by PA injected into Sprague Dawley rats that had undergone bilateral CN cut. SHH protein was quantified by semiquantitative IHC analysis in control and SHH-PA-treated tissues in order to determine if SHH-PA treatment elevated SHH protein abundance in the penis. At 2 days after CN cut, there was no change in SHH protein in the presence of SHH-PA ($n = 4$, Figure 2A, $P = 0.48$) by comparison to controls ($n = 4$). At 4 days after CN cut, SHH-PA ($n = 4$) caused a 9.3% increase in SHH protein by comparison to BSA-PA controls ($n = 3$, Figure 2A, $P = 0.06$). At

7 days after CN cut, SHH-PA (n = 4) increased SHH protein by 19% (Figure 2A, $P = 0.003$) in comparison to controls (n = 4). These results are very similar to those observed previously using Affi-Gel beads to deliver SHH protein to the corpora cavernosa to suppress apoptosis observed after CN injury [14]. When double the amount of SHH protein was added to the PA, the SHH concentration increased in the penis 30.4% at 4 days after CN cut (n = 3, $P = 0.009$) by comparison to controls (n = 3).

TUNEL Quantification of Apoptotic Index in SHH-PA and BSA-PA-Treated Corpora Cavernosa

SHH protein or BSA (control) was delivered to corpora cavernosa by PA injected into Sprague Dawley rats that had undergone bilateral CN cut. TUNEL assay was performed to determine if SHH-PA treatment could suppress CN injury-induced apoptosis in the penis. At 2 days after CN cut, there was no change in apoptotic index in the presence of SHH protein (n = 4, Figure 2B, $P = 0.20$) by comparison to controls (n = 4). At 4 days after CN cut, SHH-PA caused a 25% reduction in apoptotic index (n = 4) by comparison to BSA-PA controls (n = 3), Figure 2B, $P = 0.049$). At 7 days after CN cut, SHH-PA continued to suppress the apoptotic index by 16% (n = 3, Figure 2B, $P = 0.040$) by comparison to controls (n = 4). These results are very similar to those observed previously using Affi-Gel beads to deliver SHH protein to the corpora cavernosa to suppress apoptosis observed after CN injury [14]. When double the amount of SHH protein was added to the PA (n = 3), a 27% reduction in apoptotic index was observed at 4 days after CN cut (control = 0.230 ± 0.011 , SHH-PA = 0.165 ± 0.011 , $P = 0.008$) by comparison to controls (n = 3).

CD3 IHC Analysis of SHH-PA Tissue

In order to examine a potential immune response, IHC analysis for CD3 protein was performed in penis tissue treated with SHH-PA for 4 days (n = 2). CD3 forms part of the T cell receptor complex of a mature T lymphocyte and is considered a good, general immune response marker. CD3 protein was not observed in the corpora cavernosa of SHH-PA-treated penis (Figure 2C), indicating no immune response in the penis in the presence of SHH-PA. Spleen tissue, which was used as a positive control for CD3, showed abundant CD3 protein in ductal structures of the spleen (Figure 2C). Normal penis, without PA, was also assayed with CD3 for comparison. CD3 was not identified in normal penis tissue (Figure 2C).

Quantification of SHH Protein Diffusion from PA In Vitro

Total protein was quantified in fluid taken from on top of SHH-PA by spectrophotometric analysis at 280 nm in order to quantify the duration of PA delivery of SHH protein. Protein was quantified at 1, 3, 5, 7, 21, 29, 46, 53, 70, 95, 122, and 141 hours after the SHH-PA was formed (n = 4). Forty-two percent of SHH protein ($2.61 \mu\text{g} \pm 0.68$) came off in the first hour after PA formation (Figure 3A). By 5 hours, 50% of SHH protein ($3.10 \mu\text{g} \pm 0.58$) had eluted from PA (Figure 3A). By 21 hours, 54% of SHH protein ($3.37 \mu\text{g} \pm 0.43$) diffused from the PA (Figure 3A). By 46 hours, 60% of the protein ($3.77 \mu\text{g} \pm 0.33$) had diffused from the PA; by 95 hours, 66% ($4.10 \mu\text{g} \pm 0.29$, Figure 3A), and by 141 hours, 73% ($4.58 \mu\text{g} \pm 0.54$, Figure 3A).

Localization of Alexa-Fluor-Labeled SHH Protein Injected into the Corpora Cavernosa

Alexa Fluor 488-labeled SHH protein was injected with PA into the corpora cavernosa of the penis (n = 3) and rats were sacrificed after 3 hours in order to determine where SHH protein is incorporated in penis tissue. Fluorescent label was observed in smooth muscle lining corpora cavernosal sinuses (Figure 3B).

Localization of SHH and Patched (PTCH1) in Penile Smooth Muscle

IHC analysis of SHH and PTCH1 proteins was performed in penis tissue (n = 3) from normal adult Sprague Dawley rats in order to determine their corpora cavernosal subcellular localization. SHH protein was identified in the cell membrane of smooth muscle cells, while PTCH1 was abundant in the cytoplasm of smooth muscle cells (Figure 4).

Discussion and Conclusions

In this study, we have shown that PA technology is an effective, biodegradable, time-release method of delivering SHH protein to the corpora cavernosal smooth muscle of the penis that does not invoke an immune response by the corporal tissue. This study also shows that SHH protein is effective in suppressing CN injury-induced apoptosis as was suggested previously using Affi-Gel beads in a rat model of neuropathy [14]. The Affi-Gel bead study resulted in a 20% reduction in apoptosis in penile smooth muscle at 4 days after CN injury [14]. Using the PA methodology, a 25% reduction in apoptosis was observed in this study. When double the concentration of SHH protein was used for treatment, a 27% reduction in apoptosis was observed. An important advantage of the PA methodology is that the entire corpora cavernosa had suppressed apoptosis; while in the bead study, measurements were made within 330 μ m of beads and apoptosis increased with distance from the beads, so the entire penis did not undergo apoptosis suppression. Since the PA technology is biodegradable between 7 and 14 days after injection, this lends substantial support to the translation of this methodology to the clinic to treat prostatectomy patients. At the time of surgery, a simple injection of PA, human SHH protein and CaCl₂ could be used to suppress morphological changes in penile smooth muscle induced by CN injury during prostatectomy. One of the longstanding issues with ED development in prostatectomy patients and animal models is that although some degree of neural regeneration occurs in the CN with time, downstream changes in penile morphology caused by loss of innervation, including smooth muscle apoptosis, are irreversible once they occur [26]. This leads to a less compliant corpora cavernosa that is not able to relax to allow blood flow into the sinuses during erection. Therefore, it is imperative that new therapies target penile smooth muscle preservation so that as reinnervation occurs, the target organ is viable enough to function once neural input is reestablished.

By confocal microscopy, we have shown that in vivo SHH protein is localized in the cell membrane of smooth muscle while the receptor for SHH, Patched (PTCH1) is located in the cytoplasm of smooth muscle cells (Figure 4). Labeled SHH protein becomes incorporated in the smooth muscle lining corpora cavernosal sinuses. Protein was visualized primarily in cytoplasm of penile smooth muscle 3 hours after injection as the protein was internalized by the SHH signaling apparatus [18–20] prior to being metabolized and broken down. Therefore, a benefit of PA delivery is that SHH protein is delivered exactly where needed in the corpora cavernosa to prevent CN injury-induced apoptosis while avoiding systemic treatment. Minimal and sporadic incorporation of SHH protein was observed in the endothelium lining corpora cavernosal sinuses. This can most likely be attributed to nonspecific incorporation by endocytosis since SHH receptors have not been observed in corpora cavernosal endothelium[14]. What effect, if any, endothelial incorporation has on penile morphology remains unclear since penile morphology appeared normal after SHH treatment, other than suppressed apoptosis induction.

Our in vitro studies of SHH release from the PA suggest that SHH protein is 73% released from PA by ~6 days after gelation, although the PA itself can endure for 7 to 14 days in vivo. The conditions under which this study was performed (using Ringer's solution) mimic the in vivo conditions of serum. The durability of the PA is dependent on several factors including pH and ionic strength. Quantification of SHH protein in the corpora cavernosa

after SHH treatment by PA shows modest increases in SHH protein abundance at 4 and 7 days after PA injection. An important point to consider is that ~50% of the protein was delivered within the first 5 hours, and only a small percentage was delivered continuously after this time. Since SHH protein levels were not measured before 2 days and SHH protein is quickly broken down within the body (approximately 1 hour [27]), it would not be surprising if SHH protein were not measurably elevated. When double the concentration of SHH protein was delivered by PA, measurable SHH levels increased to 30%, suggesting further optimization of SHH levels delivered may be required for maximal apoptosis suppression. Although apoptosis suppression only increased by 2% by doubling the SHH concentration, suggesting a point of minimal return by further increasing SHH levels. It is possible to engineer the PA to be more rigid and thus have a longer SHH retention time. However, this short-term (6 days) SHH protein delivery was effective in suppressing CN injury-induced apoptosis by 25%. SHH protein is a secreted protein that has been known to diffuse over long distances and its signal transduction to be observed over an extended region beyond its localization [28]. Thus, short-term delivery of SHH protein is sufficient to suppress apoptosis, at a minimum, in the first week after CN injury. While multiple injections can be done at different times after prostatectomy, the first week after CN injury is when most of the apoptosis occurs [13], so suppression during this time is likely sufficient to prevent morphological changes in the penis. While beyond the scope of this study, further optimization is required to determine the most optimal concentration of SHH for apoptosis suppression, the maximal duration of suppression after treatment and if the apoptosis suppression is larger in a less severe CN crush model (rather than cut), which mimics the more prevalent tension and crush injuries observed during prostatectomy. A goal of this study was to determine the feasibility of using this type of delivery method in prostatectomy and diabetic patients. While there is still much work remaining regarding optimization of the methodology, PA delivery of SHH protein to the penis of prostatectomy and diabetic patients holds much promise of suppressing the smooth muscle apoptosis which leads to ED.

A potential concern of using SHH protein as a treatment in patients is the identification of upregulated SHH pathway signaling in certain cancers. One of the benefits of PA SHH delivery is that it is local, rather than systemic, and localized SHH protein treatment of the penis at the time of CN injury/prostatectomy should not affect cancer growth in other organs. Mutations in the *Shh* signaling pathway that target *Ptch1*, *Smo* and *Gli1* are associated with cancers of the prostate, skin, and esophagus [29–31]. These mutations cause continuous transcription of *Shh* targets and are not caused by an overabundance of *Shh* itself (For review, see Datta and Datta [32]). Tumor specific mutation in SHH targets including *Smo*, *Ptch1*, *Hip*, and *Gli1* (transcriptional activator of SHH) have been shown to keep the SHH pathway active in the absence of SHH ligand. However, when *Shh* is overexpressed, the tissue morphology and growth rate are normal in cancer cell lines and overexpression of *Shh* does not cause abnormally high levels of activation of *Ptch1* or *Gli1* in LNCaP cells. Thus, the use of SHH protein as a potential therapy to prevent CN injury-induced apoptosis in humans should not cause an increase in metastasized tumor growth. Although overexpression of SHH itself has not been shown to cause any adverse reaction, local rather than systemic delivery of SHH protein would avoid complications in prostatectomy populations, and thus, nanofiber delivery of SHH protein is ideal for this purpose. Patients likely to be exposed to SHH protein, as part of a putative therapy, would only be men with localized prostate cancer treated successfully by extirpative surgery. Those with residual tumor would likely not be considered candidates. While mutations which cause the SHH pathway to remain on even when SHH ligand is not bound to PTCH1 are associated with prostate cancer, it is unlikely that localized delivery of SHH protein transiently to the penis in order to suppress post prostatectomy apoptosis would affect prostate cancer growth.

In conclusion, these studies show that PA technology is effective in delivering SHH protein to the penis in an extended release manner and that SHH is effective in suppressing CN injury-induced apoptosis in the Sprague Dawley rat model. These results suggest substantial translational potential of this methodology to the clinic to suppress apoptosis in prostatectomy patients at the time of surgery and show that only a short duration of SHH treatment is required to impact the apoptotic index. Since other ED models, the BB/WOR diabetic rat which mimics type 1 diabetes in humans, exhibit a parallel decrease in SHH protein and increased apoptotic index as those observed in the Sprague Dawley CN injury model, SHH protein delivered by PA may be potentially applicable in this ED population as well to suppress apoptosis that develops with peripheral neuropathy.

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Figure 1.

(A) Structure of (C16)-V₃A₃E₃-COOH PA with and without the pyrene fluorophore. (B) 20 μ L fluorescent PA was injected into the corpora cavernosa of the penis and was visualized under dark light as a highly brightened region of the penis. (C) Fluorescent PA injection was optimized so that the PA forms a gel throughout the entire penis uniformly and was visible under dark light as a bright glow in the entire corpora cavernosa. Arrows indicate fluorescent PA. Asterisk indicates the injection site. 100X. (D) Frozen sections of penis injected with the fluorescent PA show that the PA forms in vivo as a thin lining in the sinuses of the corpora cavernosa adjacent to the smooth muscle that undergoes apoptosis with CN injury. The PA does not appear to block or impede blood flow. Arrows indicate PA. 100X. A photo of penis tissue without PA is shown for comparison. 100X. (E) PA remained abundant in the penis 7 days after injection despite the presence of shear stress caused by blood flow in the sinuses. Arrows indicate PA. 100X. (F) PA scaffold degraded between 7 and 14 days after injection. PA is visible at 14 days as a diffuse fluorescence in the corpora cavernosa (Arrow). 100X. Panel B, C, and E show the ventral aspect of the penis. Panel D and F are photos taken midway between the proximal and distal regions of the penis.

Figure 2.

SHH-PA and BSA-PA (control) were injected into the corpora cavernosa of adult Sprague Dawley rats that under went bilateral CN cut and were sacrificed at 2, 4, and 7 days after injection. (A) Semiquantitative immunohistochemical analysis for SHH protein showed a 9.3% increase in SHH protein ($P = 0.06$) at 4 days after CN injury/SHH-PA injection and a 19% increase in SHH protein 7 days after CN injury/SHH-PA injection. Photos shown were taken of 7-day tissues. Arrows indicate SHH staining. 250X. C = control. S = SHH. (B) Counting of apoptotic cells (TUNEL)/all cells (DAPI) showed a significant 25% reduction in apoptotic index at 4 days after CN injury/SHH-PA injection and a 16% reduction 7 days after CN injury/SHH-PA injection. Photos shown were taken of 4-day tissues. White arrows indicate apoptotic cells, which fluoresce green. Red arrows indicate red blood cells, which auto-fluoresce in the orange range. 250X. C = control. S = SHH. (C) Immunohistochemical analysis for CD3 in penis tissue treated with SHH-PA showed no CD3 protein, indicating no immune response of the tissue in the presence of SHH-PA. 100X. Spleen tissue, which was used as a positive control for CD3, showed abundant CD3 protein in ductal structures of the spleen (Arrow). 100X. Normal adult penis tissue (with out PA) was stained with CD3 for comparison. CD3 was not identified in normal penis tissue. CD3 positive areas fluoresce green. *Denote significant differences in SHH protein and in the apoptotic index.

Figure 3.

(A) Quantification of SHH protein release from PA in vitro. Error for each point of the graph is too small for error bars to appear in the diagram. (B) Alexa Fluor 488-labeled SHH protein was injected with PA into the corpora cavernosa of adult Sprague Dawley rats. At 3 hours after injection, fluorescent label was observed in smooth muscle lining the corpora cavernosal sinuses but not in PA only treated penis. Arrows indicate fluorescent SHH protein. An asterisk indicates a minor amount of SHH into endothelium. 400X.

Figure 4.

IHC analysis and confocal microscopy of SHH and PTCH1 localization in penile smooth muscle. SHH protein is present in the cell membrane of smooth muscle cells, while PTCH1 is abundant in the cytoplasm of smooth muscle cells. 630X and 1000X. Arrows indicate smooth muscle cells stained with SHH/propidium iodide and PTCH1/propidium iodide. Propidium iodide was used to identify the nucleus of cells.