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Engineering an immunomodulatory drug-eluting stent to treat laryngotracheal stenosis

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Abstract

Objective: Develop a drug-eluting stent construct with a reliable drug- release profile and adequate mechanical stability for a trial in a small animal model of laryngotracheal stenosis (LTS), a debilitating pathologic narrowing of the airway leading to significant shortness of breath.

Methods: Biodegradable, biocompatible stents containing 1.0% rapamycin made of PLLA-PCL (70 % Poly-L-Lactide and 30% Polycaprolactone blend) and 50:50 PDLGA (Poly(DL-lactide-co-glycolide)) were compared. Mechanical strength testing and drug elution rates using high performance liquid chromatography analysis (HPLC) was assessed. Next, efficacy of stent elution on LTS derived scar fibroblasts. Finally, stents were placed in situ in an LTS mouse model.

Results: The PLLA-PCL stent construct exhibited greater mechanical strength compared to the PDLGA stent over a 4-week period (Young's Modulus (PLLA-PCL) =13.82; Young's Modulus (PDLGA) = 4.015). Moreover, the PLLA-PCL stent showed a reliable rapamycin release profile for 6 weeks (30% elution for PLLA-PCL stents compared to <1% elution for PDLGA). Collagen 1 (p<0.05) and fibroblast cell proliferation were decreased in vitro when treated with the rapamycin stent. In vivo, the rapamycin stent reduced lamina propria thickness (p<0.05) and collagen 1 (p<0.05), collagen 3, TGF-B (p<0.05) and α -SMA (p<0.05).

Conclusions: The PLLA-PCL construct demonstrated superior mechanical strength and greater drug elution compared to PDLGA stents. We demonstrated the feasibility of testing this drug-eluting stent in vivo, showing that the rapamycin-eluting stent treats fibrosis. To our knowledge this is the first study to deploy a drug-eluting stent to treat tracheal pathology in an animal model. Optimization of a rapamycin-eluting PLLA-PCL stent for translational investigation will lead to improved treatment strategies of LTS.

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Conflicts of Interest

There are no conflicts of interest to declare.

Declarations of interest: none.

Graphical Abstract

A rapamycin-eluting PLLA-PCL stent is deployed into a diseased mouse trachea to treat laryngotracheal stenosis.

Keywords

laryngotracheal stenosis; tracheal stenosis; drug-eluting stent; PLLA-PCL; PDLGA; rapamycin

Introduction

Laryngotracheal stenosis (LTS) is a pathologic narrowing of the subglottis and trachea, leading to extrathoracic obstruction and significant shortness of breath. The pathogenesis of LTS has several etiologies, including post-intubation injury, autoimmune disease, and idiopathic. The most common etiology of LTS is iatrogenic due to post-intubation injury or post-tracheostomy injury. Iatrogenic LTS affects up to 12% of adults ^[1] as well as 11% of infants who require long-term intubation. In addition to dyspnea, scarring of the subglottis and trachea as a result of the intubation injury can lead to dysphonia and sometimes aphonia [2–5]. More critically, the luminal narrowing of the trachea can progress rapidly to airway compromise, which can result in a life-threatening event. Given the nature of this disease, up to 66% of patients require a permanent tracheostomy ^[2], which is required when patients experience acute respiratory failure from the narrowed airway. The airway prosthesis placed through the neck serves as a source of added morbidity for these patients due to foreign body reaction and mechanical irritation^[6].

The primary treatment paradigm for iatrogenic LTS is surgical and includes serial dilation, tracheal resection of the fibrotic segment, or tracheostomy. Surgical treatment for LTS can be effective, however there are risks, especially those due to infection and subsequent scarring and restenosis. Patients who are managed with serial dilations can require surgery every 4–8 months ^[6]. Tracheal resection can be curative but includes morbidity and in some

cases mortality [4]. Given the associated morbidity and mortality of surgical procedures and risks of general anesthesia, medical therapies capable of mitigating scar progression or reducing the number of surgical interventions would be valuable. The lack of medical therapies is due to our knowledge gap in the mechanism of scar formation.

LTS is a fibroproliferative disease that results from mucosal injury, usually a result of injury from an endotracheal or tracheostomy tube. In normal systems, tissue damage in the trachea during intubation results in a local inflammatory response that stimulates physiologic wound healing [7,8]. However, when this wound-healing process goes awry, an abnormal inflammatory response can lead to the deposition of pathologic scar tissue and development of LTS [9]. The injury causes permanent changes to the fibroblasts within the scar tissue, which drives continued deposition of collagen ultimately resulting in significant airway narrowing in the larynx and trachea [10, 11]. Previous investigation from our laboratory demonstrated that the hyperfunctional LTS fibroblasts are hyperproliferative, demonstrate Warburg-like physiology and have increased collagen expression [10]. Given that LTS has been shown to be mediated through an aberrant and hyper-regulated inflammatory response, therapies aimed at arresting the pro-inflammatory cascade that stimulates collagen deposition, scar formation and restenosis after a surgical intervention, present a viable strategy. Specifically, LTS has been attributed to an abnormal response of T-lymphocytes and macrophages [9, 12]. Immunohistochemical analysis from our laboratory demonstrated increased populations of macrophages and CD4 T-cells in LTS resection specimens [13, 14]. Additionally, in a mouse model of LTS, with the removal of circulating lymphocytes, the development of tracheal scar is inhibited, which implicates that treatment strategies aimed at immunosuppression may be beneficial in the management of LTS [9]. One such immunosuppressive agent that has been explored in our laboratory is rapamycin. Rapamycin inhibits mTOR signaling complex, arresting T-cell differentiation while also serving as a metabolic inhibitor [15]. In preclinical studies, rapamycin has demonstrated a robust anti-fibroblast effect and was capable of inhibiting collagen expression and deposition in LTS-derived [16].

Rapamycin is an FDA approved immunosuppressive drug, however oral rapamycin can lead to systemic side effects. Drug eluting stents have been used in the field of cardiology for many years; yet there are few studies exploring the use of a drug-eluting stent in the trachea [17, 18]. A potential method for local administration in LTS is a drug-eluting stent because it can deliver the drug directly to the diseased area and avoid systemic side effects. Airway stents are commonly used in the larynx and the trachea to mechanically manage airway stenosis and malacia [17], and drug-eluting silicone and nickel stents have been tested in animal models [17]. However, the disadvantages of such immunogenic nickel and silicone stents include increased granulation tissue and inflammation in reaction to the stent as well as difficulties in removal of the stent [19]. Biocompatible, non-inflammatory stents, with the capacity for drug-elution have been used in other organ systems to provide local therapy [20]. Such stents are made out of materials such as polycaprolactone (PCL), poly(L-lactic acid) (PLA), polydioxanone (PDA), Poly (DL-lactide-coglycolide) (PDLGA), and combined polymers such as PLLA-PCL [21]. However, biocompatible materials have not yet been tested in the treatment of airway stenosis. Given rapamycin's potential to inhibit LTS progression when given systemically, the use of rapamycin in a biocompatible, drug-eluting stent may

allow for local inhibition of the inflammation leading to airway fibrosis while avoiding systemic toxicity.

The objective of this study was to engineering a drug-eluting, biocompatible stent for the airway and test its ability to inhibit fibroblast proliferation and collagen production in vitro and its safety and efficacy in vivo. To that aim, we developed a drug-eluting biodegradable stent with sustained release of rapamycin. We showed that PLLA-PCL is a better material than PDLGA for eluting rapamycin in a reliable timescale in physiologic conditions. Additionally, mechanical testing demonstrated the PLLA-PCL rapamycin-eluting stent to be structurally stable over 8 weeks in physiologic conditions. We then tested the efficacy of this stent to inhibit LTS-scar fibroblast proliferation, collagen gene expression, functional collagen production in vitro. Finally, we miniaturized the stent and successfully placed it into a mouse trachea to demonstrate in vivo proof of concept and efficacy in reducing collagen production and decreasing fibrosis in the larynx and trachea. Using an existing murine model of LTS, we aimed to determine whether rapamycin-eluting stents placed in the mouse trachea would be feasible, safe and effective.

Methods

In Vitro Experiments

Stent Manufacturing: PLLA-PCL (70:30) (Evonik Industries AG, Darmstadt, Germany) or PDLGA (50:50 – PDS:PLGA) (Sigma Aldrich, St. Louis, MO) was dissolved and homogenized in dichloromethane (Sigma Aldrich, St. Louis, MO) in a 1:1 solution in a vacuum chamber. The solution contained 20% glycerol (Fisher Scientific, Hampton, NH) with or without 1% rapamycin (LC Laboratories, Woburn, MA). Bio-based polymers are generally plasticized (adding glycerol or water) to decrease stiffness of the construct for better biocompatibility [22]. Stents for in vitro experiments were glass-casted and left to dry in a vacuum chamber for 24 hours. Stents for in vivo experiments were casted on a 22-gauge angiocatheter and left to dry in a vacuum chamber for 24 hours. Each mouse tracheal stent was 4 mm². Total potential dosage in each stent was 3.092 µg. Therefore if 30% of the drug was released, then dosage delivered to the mouse trachea was 1.176 µg of rapamycin, assuming there is no degradation of the PLLA-PCL stent in vivo.

Stent Rheology: In order to differentiate the mechanical properties of the PLLA-PCL and PDLGA stents, stents (n=3) were compressed by a 1-N force and stiffness and elastic modulus was assessed. The 1N load was applied over the length of the stents with a 625 mm² plate mounted on an ARES G2 (TA Instruments, New Castle, DE) rotational rheometer at 37°C.

Stent Drug Elution: Stents were placed in 2 mL phosphate buffer solution (Sigma Aldrich, St. Louis, MO) and were incubated at 37°C. The solution medium was collected every day for HPLC analyses. Two milliliters of PBS was replaced for the next 24-hour period. Daily HPLC was completed for 7 days after which HPLC was conducted weekly. Rapamycin concentration in the PBS solution were determined using a HPLC standard curve for rapamycin. HPLC was conducted on a Waters 2707 autosampler (Waters, Milford, MA). The column used for separation of rapamycin was a Zorbax SD C₁₈ 4.6 cm x 250 mm

HPLC column (Agilent Technologies, Santa Clara, CA). The mobile phase contained HPLC grade water (Fisher Scientific, Hampton, NH) and acetonitrile (Fisher Scientific, Hampton, NH), (10/90 volume/volume) with a flow rate of 2.0 mL/min. The absorbance for rapamycin was measured at 280 nm. Samples were tested in triplicate. A calibration curve was made from the standard sample dilutions to determine the assay standard curve.

Human Fibroblast Culture: Human LTS-scar and normal airway fibroblasts (n=4) harvested from patients with LTS were grown to confluence, trypsinized, and plated on 6-well plates (Falcon by BD Biosciences) with 2.5×10^5 per well with fibroblast culture medium which consisted of Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (FBS; HyClone by Thermo Scientific, Logan, Utah, USA), 1000 U/mL penicillin. Informed consent was obtained from participants, as approved by the Johns Hopkins Institutional Review Board. This study was performed in accordance with the Declaration of Helsinki, Good Clinical Practice, and applicable regulatory requirements. Each patient was consented prior to any study-related procedures.

Cells were seeded in each of the following conditions in triplicate. LTS-scar fibroblasts were cultured in 3 conditions (No stent, PLLA-PCL stent without rapamycin, and PLLA-PCL stent with 1% rapamycin) in fibroblast growth media alone for 5 days in a 37°C and 5% CO₂ incubator. Patient specific normal airway fibroblasts were assessed as a control.

Human Fibroblast Proliferation: Normal and LTS-scar fibroblasts (n=4) were grown to confluence, trypsinized and plated in a 96-well culture plate (Falcon by BD Biosciences, San Jose, California, U.S.A.) with fibroblast growth medium at 100,000 cells per well in the following experimental conditions: normal fibroblasts with growth medium, scar fibroblasts in growth media, normal fibroblasts incubated with PLLA-PCL stents without rapamycin, scar fibroblasts incubated with PLLA-PCL stents without rapamycin, normal fibroblasts incubated with PLLA-PCL stents with rapamycin and scar fibroblasts incubated with PLLA-PCL stents without rapamycin. After 5 days of incubation, cells were treated with MTS Colorimetric Assay (Abcam, Cambridge, U.K.) per manufacturer protocol. Absorbance was measured with a BioTek Synergy 2 Microplate reader (BioTek Instruments, Winooski, Vermont, U.S.A.) at optical density (OD) of 490nm. Each sample was run in triplicate.

Human Fibroblast Quantitative Gene Expression Analysis: RNA was extracted from cell cultures (n=4) seeded at the above mentioned concentrations (2.5×10^5 per well) using the RNeasy MicroKit as a part of the Allprep RNA/protein kit (Qiagen, Valencia, California). RNA was quantified using a Nanodrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA) and then used to create cDNA. Gene expression was assessed by quantitative real-time polymerase chain reaction (PCR) with a StepOnePlus Real Time PCR system (Life Technologies, Carlsbad, California) and Power SYBR Green Mastermix (Life Technologies). Complementary DNA samples were diluted 1:30, and 1 mL was combined with 10 mL of Mastermix, 8 mL of ddH₂O, and 0.5 mL of 10mM forward and reverse primer for the gene of interest (total volume of 20 mL). Each reaction well underwent 40 cycles of a denaturation step at 95°C for 15 seconds, followed by annealing and extension at 60°C for 60 seconds. The cycle threshold for gene product detection (CT) of the gene of interest was normalized against the CT value for the reference gene β -actin (DCT) for all

samples and then compared with normal controls (DDCT). Gene expression is presented as the relative fold change calculated by 2^{DDCT} . All samples were investigated in triplicate. Changes in gene expression of the fibrosis markers, collagen 1 and collagen 3 were assessed using quantitative Real-time PCR [23].

Statistical Analysis: Results are displayed as mean and standard error. Differences between treatment and control groups were compared using a parametric, paired T-test with $p < 0.05$ being accepted as significant. Data analysis was performed using Prism GraphPad statistical software (La Jolla, CA).

In Vivo Experiments

LTS and stent placement in mice: Stents for use in a mouse model of LTS were molded around a 22-gauge angiocatheter using a PLLA-PCL polymer solution (which was doped with rapamycin to form a 1% rapamycin solution for rapamycin-eluting stents). We determined that the amount of rapamycin in each stent did not exceed the toxic levels (6 mg/kg/day) as described by similar rapamycin-eluting stents for coronary arteries [24]. Each miniaturized stent placed in the trachea contained 0.733 $\mu\text{g}/\text{mm}^2$ of rapamycin. Mice were purchased were 10 week old males (C57BL/6) obtained from Charles Rivers Laboratories. Mice were randomized to three treatment groups (n=8): 1) stent with rapamycin, 2) stent without rapamycin and 3) positive control trachea without stent. Mice were placed under anesthesia and LTS was induced [25]. Mice underwent chemomechanical injury of the trachea using a bleomycin-coated wire brush to induce LTS as per the original study by Hillel et al (2014). Mice were then randomized to the stent with rapamycin or stent without rapamycin had the appropriate stent placed transorally under direct visualization (by performing a transcervical incision to see the trachea) of tracheal disruption, using the Seldinger technique [25]. We placed the stents in the mouse trachea, confirming visualization of the stent transcervically. Mice were monitored for 4, 7, and 14 days, after which they were sacrificed. All experiments were performed in compliance with the Johns Hopkins School of Medicine policies on animal use and ethics (MO18M124). This study was approved by the Johns Hopkins University Animal Care and Use Committee.

Histologic Quantification of Fibrosis: Mouse tracheas that were treated with rapamycin-eluting stents and those that were not treated with any stents were harvested and fixed in 10% formalin overnight. Tissue were embedded in paraffin and 5 μm sections were cut axially through the proximal trachea. H&E stained sections were used to measure the thickness of the lamina propria as previously described. This is a validated methodology for assessing tracheal stenosis in an animal model [23]. Microscopic images of the stained sections were obtained with a microscope (AX10; Zeiss) at $20 \times$ magnification (n=4).

Stent Biocompatibility Testing: Sections were prepared and processed as previously described [14]. First, sections were de-paraffinized in xylene twice for 5 minutes. Then, samples were hydrated with 100% ethanol twice for 3 minutes, followed by hydration with 95% ethanol for 1 minute. Then, de-paraffinized slides in a histology staining rack with an antigen retrieval buffer. Staining racks were then placed in a vegetable steamer with boiling water. We kept the staining racks in the steamer for 20 minutes, after which, we removed the

stained racks from the steamer and discarded the buffer inside and added PBS. We then put the slides into a wet staining box, and incubated slides with 1 mL of PBS for 1 minute. PBS was then discarded and slides were incubated with DMEM with 10% FBS for 30 minutes to block non-specific binding of antibodies. Then, DMEM was discarded and slides were incubated with a mixture of two primary antibodies (for CD3, CD4, CD20 and F4/80) diluted in DMEM with 10% FBS. Slides were then covered with parafilm and incubated overnight at 4°C in a dark room. The next day, slides were washed three times in PBS for 5 minutes per wash. Slides were then incubated with the mixture of two secondary antibodies diluted in DMEM with 10% FBS for 0.5 to 1 hour at room temperature in the dark. Slides were then washed with PBS for 5 minutes, three times in the dark. Samples were mounted on slides with cover slips and a drop of mounting medium with DAPI. Slides were stored in the dark at -20°C or 4°C. Slides were read on a confocal microscope (Zeiss LSM 510 Meta Confocal Microscope). Immunohistochemical staining of inflammation markers CD4, CD3, CD20, F4/80 were assessed for T-cell activity and macrophage activity and compared between tracheas from mice that underwent mechanical brush injury (positive controls), uninjured tracheas from mice that underwent mock surgery (negative controls) and tracheas from mice that had plain stents placed.

Primary antibody	Rabbit anti-CD3	AbCam Ab5690
Primary antibody	Rat antiF4/80	Biolegend 123101
Primary antibody	Rabbit anti-CD20	Reprokine RPA11836
Primary antibody	Rat anti-CD4	Biolegend 100402
Secondary antibody	Goat anti-Rat-633	Life Technology A21094
Secondary antibody	Goat anti-Rabbit-488	Life Technology A11008

Inflammatory and Fibrotic Effect of Rapamycin-Eluting Stent by Gene

Expression—RNA was extracted from tracheal tissue, which was homogenized and processed to perform quantitative PCR as described above. Gene expression of TNF- α , INF- γ , Arg-1, IL-4, IL-8, IL-1b, and CD11b were assessed for inflammation. Gene expression of Col1, Col3, α -smooth muscle actin (α SMA), fibronectin, and transforming growth factor β (TGF β) were measured to assess fibrosis.

PCR Primer Sequences

Gene	Forward Primer (Human)	Reverse Primer (Human)
Col-1	5' -GAGGGCCAAGACGAAGACATC-3'	5' -CAGATCACGTCATCGCACAAC-3'
Gene	Forward Primer (Mouse)	Reverse Primer (Mouse)
INF- γ	5'-ATGAACGCTACACACTGCATC- 3'	5'-CCATCCTTTTGCCAGTTCCCTC- 3'
TNF- α	5'-CTGAACCTCGGGGTGATCGG-3'	5'-GGCTTGCTACTCGAATTTGAGA-3'
IL-4	5'-GGTCTCAACCCCAAGTCTAGT-3'	5'-GCCGATGATCTCTCTCAAGTGAT-3'
IL-8	5'-TCGAGACCATTTACTGCAACAG-3'	5'-CATTGCCGGTGGAATTCCTT-3'
CD11b	5'-GGGAGGACAAAACTGCCTCA-3'	5'-ACAACTAGGATCTTCGCAGCAT-3'

Gene	Forward Primer (Human)	Reverse Primer (Human)
Arg-1	5'-CTCCAAGCCAAAGTCCTTAGAG-3'	5'-GGAGCTGTCATTAGGGACATCA-3'
Col-1	5'-CTGCGGTTTCAGGTCCAAT-3'	5'-TTCCAGGCAATCCACGAGC-3'
Col-3	5'-CTGTAACATGGAACTGGGGAAA-3'	5'-CCATAGCTGAAGTGAACCACC-3'
α -SMA	5'-CCCAGACATCAGGGAGTAATGG-3'	5'-TCTATCGGATACTTCAGCGTCA
FBN	5'-GGACGCCAATTGGAGGCT-3'	5'-CTTTCAGCGCATCGTGTCT-3'
TGF- β	5'-GTTCTTCAATACGTCAGACATTCG-3'	5'-GTAACGCCAGGAATTGTTGCTA-3'

Statistical Analysis: Results are displayed as mean and standard error. Differences between treatment and control groups were compared using a non-parametric, unpaired T-test with $p < 0.05$ being accepted as significant. Data analysis was performed using Prism GraphPad statistical software (La Jolla, CA).

Results

PLLA-PCL stents have greater mechanical stability and drug-elution than PDLGA stents—First, our objective was to determine the appropriate stent material for the airway. We compared the rapamycin elution and mechanical strength of two biodegradable and bioresorbable polymers that have been used to make coronary artery stents: PDLGA and the composite material PLLA-PCL. To accomplish this, we constructed the stents containing rapamycin in a 2-D sheet structure using glass-casting. These stents were incubated in PBS and the amount rapamycin elution into the PBS over time was detected using serial HPLC measurements.

Analysis of the HPLC results (Figure 1a) demonstrated that the PLLA-PCL stents ($n=3$) showed a reliable rapamycin release profile for 6 weeks with 30% rapamycin elution for PLLA-PCL stents compared to $<1\%$ rapamycin elution for the PDLGA stents ($n=3$). Additionally, we observed the structure of the 2-D stents over time in the PBS solution. The PDLGA stent became brittle and degraded over 3 weeks in PBS, whereas the PLLA-PCL stent maintains qualitative structure integrity over 3 weeks in PBS.

To determine and compare the mechanical strength of the stents, we next constructed 3-D cylindrical PLLA-PCL ($n=3$) and PDLGA stents ($n=3$) using glass casting (Figure 1b). Compression studies were completed using a rheometer to compare the mechanical compressive strength of the two stents. Figure 2c and 2d show the deformation of the PLLA-PCL and PDLGA stents when subjected to a 1N load. The PLLA-PCL stent construct exhibited greater mechanical strength compared to the PDLGA stent over a 3-week period (Young's Modulus (PLLA-PCL) = 13.82; Young's Modulus (PDLGA) = 4.015) (Figure 1c, d). Furthermore, when PLLA-PCL stents were subjected to compressive loads, they regained their shapes as loads were removed. In their cylindrical form, we observed that the PLLA-PCL stent also maintains its cylindrical shape without collapsing, whereas, over a period of 3 weeks the PDLGA stent slowly collapses when incubated in PBS solution (Figure 1b). Three weeks post-incubation, the PLLA-PCL stent has reduced stiffness of Young's Modulus = 7.55 Pa but the PDLGA stent flattens, and loses its cylindrical stability. Hence, it

was determined that PLLA-PCL is the superior polymer to release rapamycin as it maintains mechanical stability in biological medium over a period of at least 3 weeks.

Rapamycin-eluting stent inhibits fibrosis in vitro—Having determined the optimal polymer material and having established that the stent elutes rapamycin in a reproducible manner, drug-eluting stents were subsequently co-cultured with fibroblasts derived from scar trachea (n=4) and normal areas of the trachea (n=4) to determine whether the eluted rapamycin would have a treatment effect on LTS-scar fibroblast proliferation and collagen production. Compared to collagen 1 gene expression in LTS-scar fibroblasts treated with plain stents (fold change: 2.38x), LTS-scar fibroblasts incubated with rapamycin-eluting stents had reduced collagen 1 expression (fold change: 0.75x, $p<0.05$) in vitro (Figure 2a).

PLLA-PCL stent placement is feasible in vivo—Stents were successfully placed into mice. Figure 3a and 3b demonstrates the technique of placing a tracheal stent while Figure 3c is an axial rendering depicting the stent within the trachea. Stent placement was confirmed grossly (Figure 3d) and then confirmed endoscopically using a sialendoscope. Endoscopic imaging also demonstrated luminal patency of the stent in situ. Figure 3e shows the mouse trachea (with the tracheal rings being visible) without the stent and Figure 3f shows placement of the stent within the trachea. Of 24 stented mice, 16 mice survived after 7 days.

PLLA-PCL stent has minimal inflammatory response in vivo—To assess in vivo inflammatory response to the PLLA-PCL stent material, we compared the inflammatory response in LTS-mice tracheas (positive control), uninjured mice tracheas (negative control), and uninjured mice tracheas with a PLLA-PCL stent (without rapamycin). LTS mice tracheas (positive controls) revealed B-lymphocytes (CD20 staining in green), T-lymphocytes (CD3 in green and CD4 in red), and macrophages (F4/80 in red) (Figure 4A and Figure 4D). Uninjured mouse tracheas had no B-lymphocyte, T-lymphocyte, or macrophage expression. Similarly, when the plain PLLA-PCL stent was placed in uninjured mouse tracheas, there was no B-lymphocyte, T-lymphocyte, or macrophage expression.

Rapamycin-eluting stent mitigates fibrosis in vivo—Having determined that the PLLA-PCL stent does not induce a foreign body reaction in the trachea, we next determined if the rapamycin-eluting PLLA-PCL stent had an effect on LTS (as measured by lamina propria thickness). Tracheas in normal mice had a lamina propria thickness of 40 μm [25]. Untreated LTS mice trachea (n=3), had increased lamina propria thickness and a consequently, a narrowed tracheal lumen (Figure 5a) when compared with rapamycin-eluting stent treated mice (n=3) (Figure 5b). In determining whether there was a mechanical effect through an outward radial force of the stent on the trachea, we did a histologic examination of a mouse trachea that was treated with a stent without rapamycin (Figure 5c), which does show some reduction in the stenosis of the trachea. The average thickness of the lamina propria for tracheas treated with rapamycin stents is 34.24 μm less ($p<0.01$) than those without stent treatment (Figure 5d).

Biological impact of rapamycin-eluting stent in vivo—Next, we assessed whether the PLLA-PCL stent with rapamycin reduced acute inflammation when compared to the

plain stent. There was a significant decrease in gene expression for Inf- γ (fold change: 1x to 0.28x p<0.05), CD11b (fold change: 1x to 0.11x p<0.05), Arg-1 (fold change: 1x to 0.17x p<0.05) and IL-1b (fold change: 1x to 0.08x p<0.05) (Figure 6a) in mice that had been treated with the rapamycin-eluting stent. Fibrosis gene markers Col1 (fold change: 1x to 0.24x p<0.05), α -SMA (fold change: 1x to 0.03x p<0.05), and TGF- β (fold change 1x to 0.05x p<0.05) were decreased in mice treated with the rapamycin-eluting stent compared to the plain PLLA-PCL stent (Figure 6b).

Discussion

We report the design and optimization of a biodegradable, biocompatible stent with sustained drug elution to treat tracheal stenosis. Initial in vitro testing demonstrated PLLA-PCL stents were superior to PDLGA stents in drug elution and mechanical stability. Drug elution from the PLLA-PCL stent was reliable over a period of 30 days in physiologic conditions (and using a physiologically compatible rapamycin dosage). Finally, in vitro testing showed that the rapamycin-eluting PLLA-PCL stents reduced and collagen 1 gene expression with a trend toward reduced LTS-scar fibroblast proliferation, justifying in vivo translation. PLLA-PCL stents were then manufactured to be placed in the mouse trachea and shown to be biocompatible with minimal inflammatory response. Finally, we deployed the rapamycin-eluting PLLA-PCL stents in our murine model of LTS and demonstrated treatment effect in vivo with reduced lamina propria thickness compared to untreated mice, and reduced acute inflammation and reduced fibrosis gene expression compared to LTS mice treated with a plain stent.

The PLLA-PCL stent was superior and allowed for consistent and sufficient delivery to produce an anti-fibrotic effect in vitro. PLLA-PCL and PDGLA (50:50 mix of PLGA and PDS) were chosen as potential candidates for the stent polymer in this study because they are biocompatible materials and are among the biodegradable polymers approved by the US Food and Drug Administration [24]. Biopolymers containing drugs have been shown to have three stages of drug release: an initial burst of drug release, diffusion-related drug release and degradation-related drug release. Having a predictable drug release profile is important in order to determine the clinical effects on the patient at pre-established time points. We showed that the PLLA-PCL material's initial burst of drug release was reproducible and its total rapamycin release percentage did not exceed the toxic amount of 6 mg/kg (as demonstrated by animal testing) [24]. Over the remaining weeks, the PLLA-PCL stent demonstrated diffusion and degradation-related drug release which allowed for the dose delivery of rapamycin to murine trachea, an important feature for clinical efficacy. Our results also showed PLLA-PCL's mechanical stability in physiologic medium over 3 weeks. Through rheologic compression studies, we showed that the PLLA-PCL stent can regain its shape after compression, which demonstrates its mechanical flexibility and shape integrity without resulting in stress fractures. These mechanical characteristics are critical for the stent when placed in the airway in being able to adapt to and resist changes in tracheal shape without exerting excess force on the trachea itself.

Next, we showed that in vitro, the rapamycin-eluting stent decreased collagen production in cell lines (normal fibroblasts and LTS-scar fibroblasts) derived from normal and scarred

tracheal tissue. The biological impact of the rapamycin was appreciated through gene expression data, which showed decreased collagen 1 expression. Additionally, there was downward trending LTS-scar fibroblast proliferation in cells treated with the rapamycin stent, which corroborates with the results from Namba et al ^[16], which showed that incubating fibroblast cell cultures with rapamycin decreased scar production. Taken together, these results validated the testing of a rapamycin-eluting stent in an in vivo model.

This is the first time, to our knowledge, a PLLA-PCL tracheal stent was deployed in a mouse model. Although other materials were used in studies with rabbit and canine models [26, 27, 28], PLLA-PCL chosen for this study because it causes little inflammation in the native tissue ^[29]. Moreover, the rabbit and canine models used in the past do not use the LTS disease model to test the effects of a DES. Further, previous studies have placed stents surgically inside the trachea. By contrast, in this study, the endobronchial insertion of the PLLA-PCL stent in the mouse was modelled after how airway stents are placed clinically. Clinical application of stents is performed transorally with the stent spanning the stenosis and verified endoscopically. In these murine experiments the stent was similarly placed transorally after LTS induction ^[25] with gross visualization through the translucent mouse trachea to verify placement. After placement, the PLLA-PCL stents remained in place for up to 3 weeks without degrading and without obstructing the tracheal lumen.

In vivo, stent biocompatibility was verified through immunohistochemical analysis which showed that the stent caused no foreign body reaction and minimal inflammation in the trachea. Day 7 was chosen as an endpoint for evaluating for foreign body reaction because the pro-inflammatory response capable of creating fibrous capsules is regulated by M1 macrophages, whose presence peaks at 4–7 days post-implantation. The absence of T-lymphocytes (CD3, CD4) and B-lymphocytes (CD20) as well as macrophages (F4/80) demonstrate a lack of acute inflammatory response or foreign body reaction to the PLLA-PCL stent after one week in situ. This study shows that the advantage of using this PLLA-PCL biomaterial to treat LTS is that it does not further increase granulation tissue in the trachea, which can worsen the problem of LTS. The biocompatibility of this stent is an advantage over drug-coated metallic stents, which frequently generate acute granulation tissue within the trachea as foreign body response to the stent ^[19]. Further studies investigating chronic inflammation of our following long-term implantation of our PLLA-PCL material are underway.

Having established safety of stent placement and stent biocompatibility in mice, we next confirmed that the rapamycin eluting stent was efficacious. The reduction in lamina propria thickness, decrease in acute inflammation and decrease in fibrosis gene expression demonstrate that the rapamycin-eluting stent is capable of treating LTS in vivo and represents a promising clinical application to treat airway scar. We used the validated outcome of lamina propria thickness to demonstrate the treatment effect of the stent on the fibrotic trachea ^[25]. We recognized that the outward radial mechanical force of the PLLA-PCL stent would be a significant contributing factor. Thus, we completed a qualitative histologic analysis of the effect of the PLLA-PCL stent without rapamycin on mice tracheas with LTS which showed that there was some reduction of stenosis when compared to tracheas that had not been treated with stents. Moreover, the advantage of the PLLA-PCL

stent with the mechanical characteristics described is that it is able to be manipulated to be deployed without irritation to the tracheal or oropharyngeal mucosa. We next determined the inflammatory and fibrotic effect of the rapamycin-elution on the diseased trachea by directly comparing the effect of the drug-eluting stent with the plain stent. The rapamycin-eluting stent had both an anti-fibrotic effect, with a reduction in Col-1, TGF- β , and α SMA gene expression, and anti-inflammatory effect, with a reduction in IL-1, INF- γ , Arg1, and CD11b, revealing its biological effect in addition to a mechanical effect. We recognize we placed the rapamycin-eluting stents immediately after LTS induction in mice, rather than allowing the stenosis to develop after injury and then treating it with the stent. While stents were not placed in the same timeline as they would be clinically, this study is a proof-of-concept study that shows that the stents are able to be successfully placed in the mice trachea and are efficacious in reducing fibrosis. We also recognize that there may be some limitations to the production of a biodegradable stent (without a metal or silicon base) in mass production when translated to the clinical level. Using glass casting or molding to construct the stent can be further explored in future studies to facilitate the ease of translating the stent production to a larger scale. Moreover, as future studies are completed in larger animal models, determining the cost-effectiveness of constructing and using rapamycin-eluting stents compared to existing treatment methodologies can be explored. Additionally, in these larger animal models, we can further test the stability of the stent in the trachea before and after placement in the trachea. Finally, in a previous study [21] with PLLA-PCL blends of 85/15 or 90/10 have showed >30% release of drug (paclitaxel) in 20 days. It is possible the slower rate of rapamycin elution in our 70/30 PLLA-PCL stent could be attributed to reduced PLLA component in the composite material. As the percentage of PLLA increases in the composite, there is a faster degradation rate (and thus drug elution) due to the amorphous structure of PLLA. In future studies, we intend to further optimize and control the rapamycin release profile by increasing the PLLA concentration in the stent.

The versatility of the PLLA-PCL stent as a drug-delivery method translates to multiple clinical applications. The current PLLA-PCL stent polymer formula can be modified to deliver other drugs as potential immunotherapies to decrease scar proliferation in the trachea. Engineering a miniaturized stent for use in a murine disease model optimizes the experimental flexibility in mice with testing in genetically modified mice, and allows comparison between large experimental cohorts. We chose to do a mouse model for this study because of several advantages the mouse model offers for studying the effect of the drug eluting stent in treating LTS. First, mouse models can be genetically modified to generate mice with genetic proclivity to forming scar. Second, in using a mouse model, it is easier to study the effects of the drug-eluting stent when comparing diseased and healthy mice through either induction of the LTS state with chemomechanical brush injury or genetic modification. Third, there is a cost-effectiveness and reproducibility of the mouse model. Future studies include mice survival experiments which include short term (3 weeks) and long-term survival (6 months). After completing mice survival studies, we plan to test the efficacy of the rapamycin-eluting stents in a larger animal model prior to clinical translation.

Conclusion

In summary, we present a rapamycin-eluting stent that had sustained drug release, is mechanically stable in vitro and in vivo and has proven to be effective in reducing stenosis in an LTS mouse model. We first showed that the PLLA-PCL stent showed reliable rapamycin elution and mechanical durability in physiological conditions compared to PDLGA stent. In vitro, the rapamycin eluting PLLA-PCL stent reduced scar and normal fibroblast proliferation and collagen gene expression. In vivo, rapamycin stents were efficacious at reducing fibrosis while generating an unremarkable inflammatory response. This highlights the advantage of the biomaterial PLLA-PCL's biocompatibility, biodegradability, and mechanical strength in treating laryngotracheal stenosis. We believe that this stent can be of significant clinical therapeutic use and can also serve as an adaptable drug-delivery tool that can be used to test multiple immunotherapies in a mouse disease model.

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Figure 1:

Stent degradation, rapamycin elution and mechanical compression of stent. (A) The PLLA-PCL stent (n=3) elutes 30% of its initial drug load in 20 days, whereas the PDLGA stent (n=3) elutes <1% of its initial load over the same period of time. (B) The PDLGA stent degrades in physiologic conditions after 21 days of incubation whereas the PLLA-PCL stent maintains its structural integrity. (C) At 0 weeks of incubation in physiological conditions, the PLLA-PCL stent has a greater Young's Modulus of 13.55 Pa compared to the PDLGA stent of 4.90 Pa. (D) Three weeks post-incubation at 37°C, the PLLA-PCL stent has reduced stiffness of Young's Modulus= 7.55 Pa but the PDLGA stent flattens, and loses its cylindrical stability. Rheologic measurements were done at 37°C.

Figure 2:

In vitro experimentation with rapamycin-eluting stents. Plain stents without rapamycin and rapamycin-eluting stents (RES) were incubated scar fibroblasts (SF) from scarred areas of the trachea (n=4). Gene expression is presented as relative fold change calculated as the cycle threshold (CT) normalized against β -actin and compared with expression from normal airway fibroblasts (NF) (2^{-CT}). (A) For scar-derived fibroblasts, incubation with rapamycin decreased COL1 expression from 1x to 0.75x. (B) MTS assay to assess fibroblast cell activity as an indicator of cell proliferation shows reduced cell activity in cultures treated with rapamycin stents by 10%.

Figure 3:

In vivo experimentation with rapamycin-eluting stents. Schematic of stent placement in mouse trachea. (A) Animated schematic of stent placement within mouse trachea orally. The stent is delivered on a 22 gauge angiocatheter into the trachea. (B) The stent is placed properly in the tracheal lumen while grasping the trachea to anchor the stent above the level of the carina, below the glottis and the angiocatheter is pulled out. (C) Animated representation of cross-section of the trachea with stent inside lumen. Stent is gray. Red dots represent how we expect rapamycin to be radially eluted into the surrounding scar tissue

radially from the stent. (D) Gross image of mouse trachea with stent placed (outlined in black). Endoscopic imaging confirms the placement of the stent in the tracheal lumen (E), compared to the tracheal lumen with the cartilaginous rings (F). (G) Stent in situ after placement. (H) On the left is the resected trachea from mouse after 3 weeks of incubation. On the right is the stent, removed from the trachea. There is little deterioration of the stent in 3 weeks in vivo.

Figure 4:

Biocompatibility of PLLA-PCL stent with trachea. Representative trachea of mouse that was injured through mechanical disruption of the trachea with PBS (n=3) at day 7 shows little CD4 (red), CD20 (green) in panel A, F4/80 (red) and CD3 (green) activity in panel D. Representative trachea of mouse that underwent mock surgery without mechanical disruption (n=3) stained for CD4 (red), CD20 (green) in panel B and F4/80 (red) and CD3 (green) in panel E. Representation trachea of mouse in which a plain PLLA-PCL stent (n=3)

was transorally placed stained for CD4 (red), CD20 (green) in panel C and F4/80 (red) and CD3 (green) in panel F.

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Figure 5:

Rapamycin-eluting PLLA-PCL stent decreases lamina propria thickness. (A) Representative histology of mouse trachea without rapamycin-eluting PLLA-PCL stent. (B) Representative histology of mouse trachea with rapamycin-eluting stent at day 7. (C) Representative histology of mouse trachea with a stent lacking rapamycin. (D) Lamina propria thickness (μm) of mouse tracheas treated with rapamycin-eluting stents (n=4) compared to tracheas without rapamycin-eluting stents (n=4) shows a reduction of 38.24 μm in thickness of trachea treated with rapamycin-eluting stent.

Figure 6:

Biological effect of rapamycin-eluting stent in vivo. (A) Fold change of genes expressing proteins leading to for acute inflammation decreased on day 7 after implantation (Inf- γ , TNF- α , IL-4, IL-8, CD-11b, Arg-1, IL-1b) in mice that were treated with the rapamycin-eluting stent (n=3) when compared to mice that were treated with the plain stent (n=3). Gene expression is presented as relative fold change calculated as the cycle threshold (CT) normalized against β -actin and compared with expression from tissue from mice that had plain stents placed (2^{-CT}). There was a significant decrease in gene expression for Inf- γ by 72%, CD11b by 89%, Arg-1 by 83% and IL-1b by 92%. (B) Collagen 1, Collagen 3, α -SMA, TGF- β and fibronectin gene expression was reduced (by 75%, 61%, 83%, 94%, 37%, respectively) in mice treated with rapamycin-eluting stent (n=3) day 4 after placement when compared to mice treated with the plain stent (n=3).