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Down-regulating ERK1/2 and SMAD2/3 phosphorylation by physical barrier of celecoxib-loaded electrospun fibrous membranes prevents tendon adhesions

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ABSTRACT

Peritendinous adhesions, as a major problem in hand surgery, may be due to the proliferation of fibroblasts and excessive collagen synthesis, in which ERK1/2 and SMAD2/3 plays crucial roles. In this study, we hypothesized that the complication progression could be inhibited by down-regulating ERK1/2 and SMAD2/3 phosphorylation of exogenous fibroblasts with celecoxib. Celecoxib was incorporated in poly(L-lactic acid)-polyethylene glycol (PELA) diblock copolymer fibrous membranes via electrospinning. Results of an *in vitro* drug release study showed celecoxib-loaded membrane had excellent continuous drug release capability. It was found that celecoxib-loaded PELA membranes were not favorable for the rabbit fibroblast and tenocyte adhesion and proliferation. In a rabbit tendon repair model, we first identified ERK1/2 and SMAD2/3 phosphorylation as a critical driver of early adhesion formation progression. Celecoxib released from PELA membrane was found to down-regulate ERK1/2 and SMAD2/3 phosphorylation, leading to reduced collagen I and collagen III expression, inflammation reaction, and fibroblast proliferation. Importantly, the celecoxib-loaded PELA membranes successfully prevented tissue adhesion compared with control treatment and unloaded membranes treatment. This approach offers a novel barrier strategy to block tendon adhesion through targeted down-regulating of ERK1/2 and SMAD2/3 phosphorylation directly within peritendinous adhesion tissue.

1. Introduction

Adhesion formation after tendon injury poses a major clinical problem [1]. It not only causes gliding dysfunction and pain but also requires complicated re-operative surgery. At present, the methods of prevention mainly contain drugs [2,3], barriers [4–7], new rehabilitation protocols [8,9] and the combination [10,11], among which, the combination of barriers and drugs were mainly studied. The methods above usually prevent adhesions by inhibiting the inflammatory reaction or isolating invasion of exogenous cells from the surrounding tendon sheath. However, fewer researches focused on the mechanism of alleviating adhesions intrinsically.

The mechanism of adhesion formation remains unknown. Current hypotheses involve cellular proliferation, vascularization, inflammatory response, synthesis of collagen and new extracellular matrix, and blood vessel in-growth [12], in which, the migration and proliferation of fibroblasts and excessive collagen synthesis are important factors [13–16]. Excessive fibroblast proliferation and collagen synthesis can form a dense layer of connective tissue, which is called peritendinous adhesions, between the tendon and the surrounding tissue. Therefore, the adhesion formation should be prevented via inhibition of fibroblast proliferation and collagen synthesis.

Celecoxib, a kind of selective nonsteroidal anti-inflammatory drugs (NSAIDs), have been found to suppress fibroblast proliferation and collagen expression by inhibiting ERK1/2 and SMAD2/3

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phosphorylation of the cells [17]. And previously, we demonstrated that extra-cellular signal-regulated kinase (ERK)2 played crucial roles in reducing collagen expression and proliferation of rat joint adhesion tissue fibroblasts [18]. It was also found that local delivery of ERK2 small interfering RNAs to silence ERK2 expression can ameliorate tissue adhesion formation effectively and safely [19]. Meanwhile, celecoxib has been used to prevent joint and intra-abdominal adhesion formation [20,21]. As tendon adhesions is a type of tissue adhesions, therefore, it is expected that, celecoxib can be used to inhibit tendon adhesions. Currently, owing to the rapid clearance and potential side effects of NSAIDs which were given by oral administration, they alone cannot completely prevent adhesions. As a result, to address these problems and to avoid adverse side effects, an advanced and topical administration route is required.

Recently, a lot of barriers such as physical membranes and gels have been used to inhibit extrinsic healing, which is considered as an important progress in tissue adhesions. The electrospun fibrous membranes, as a physical barrier, are applied to anti-adhesions and drug delivery owing to their high porosity, very small pore size and large surface area-to-volume ratio [22]. In addition, this approach of drug delivery can avoid the adverse gastroenteric reaction of NSAIDs and repeated local injections. We have previously successfully used electrospun fibrous membranes with ibuprofen or hyaluronic acid to prevent the tissue adhesions due to their better flexibility, favorable hydrophilic properties, drug delivery and anti-adhesions ability [10,23]. However, these studies only show the effects of anti-adhesions superficially, and the mechanism of prevention of tendon adhesions still remains unknown.

In this study, celecoxib-loaded electrospun PELA fibrous membranes were tested for the effects of suppressing fibroblast proliferation and collagen expression of adhesion formation *in vitro* and *in vivo* by inhibiting ERK1/2 and SMAD2/3 phosphorylation and the ability to minimize adhesion formation. Thereby, we indicated the mechanism of tendon adhesions and discovered a novel method of anti-adhesion preliminarily.

2. Materials and methods

2.1. Materials

2.1.1. Electrospinning of nanofibrous membranes

All reagent grade chemicals and solvents except otherwise indicated were purchased from GuoYao Regents Company. Electrospinning was carried out according to our previous report [24]. First, 1 g PELA (Mw = 40 kDa, E/L = 10/90, Mw/Mn = 1.56) and celecoxib (TSZ, USA) were completely dissolved in a solvent containing 2.1 g dichloromethane(DCM) and 1.1 g dimethylformamide (DMF) for preparing celecoxib-loaded PELA electrospun fibrous membranes. Then, we used a 0.7 mm diameter needle fitted to a 2.0 ml glass syringe and a syringe pump which the solutions containing 2%, 6%, or 10% (0.02 g, 0.06 g and 0.1 g) celecoxib were drawn into for preparing 2%-PELA, 6%-PELA and 10%-PELA membranes, respectively. A high-voltage power supply provided a 15 kV voltage difference between the needle tip and a collector (grounded aluminum foil). The electrostatic force drew the polymeric solution from the needle tip to reach the collector that was placed 15 cm from the needle tip. The flow rate of the polymer solution was controlled at 3.0 ml/h. The fabricated fibrous membranes were dried overnight in a vacuum oven before the test.

2.1.2. Characterization of the electrospun fibrous membranes

The morphology of the membranes was observed by scanning electron microscopy (SEM, FEI Quanta 200, Eindhoven, Netherlands). The apparent porosity was calculated by obtaining at least 5 images of each sample according to previously published methods [25]. The mean diameter was calculated by measuring at least 200 random fibers from 20 images with Photoshop 8.0.

The wettability of different surfaces was determined using a sessile drop contact angle system with a Krüss GmbH DSA 100 Mk 2 goniometer. The water contact angles were measured after 10 s at 25 °C and calculated by image processing of sessile drop profiles with DSA 1.8 software.

The mechanical properties were measured using rheometer (Instron 5567, Norwood, MA). The test strips (70.0 × 7.0 × 0.6 mm) was vertically mounted on two mechanical gripping units at both ends, leaving a 3 cm gauge length for mechanical loading. Load-deformation data were recorded at a stretching speed of 0.5 mm/s. The ultimate tensile strength and Young's modulus were obtained from the stress–strain curves ($n = 5$).

2.1.3. Drug release study

The test specimens (20 × 20 mm; total mass ≈ 50 mg) of celecoxib-loaded PELA fibrous membranes were immersed in 20 ml phosphate buffered saline (PBS, 154 mM, pH 7.4). The suspension placed in 50 ml centrifugal tube was maintained in a thermostated shaker (Thermo, USA) at 37 °C with 100 cycles/min. 5.0 ml released buffer was removed from the centrifugal tube and 5.0 ml fresh PBS was replaced at different time points. The quantity of released celecoxib from celecoxib-loaded PELA fibrous membranes in the release buffer was measured by ultraviolet–visible spectrophotometry (UV-2550, Shimadzu, Japan) at 249 nm. A linear correlation ($R^2 = 0.9917$) was obtained using absorbance versus celecoxib concentration of standard samples (0, 2, 4, 6, 8, 10 and 14 μg/ml). Based on the initial weight of celecoxib incorporated into the electrospun fibrous membrane, the percentage of released drug from the membranes was calculated.

2.2. In vitro cell experiment

2.2.1. Isolation and culture of tenocytes and dermal fibroblasts

Tendon tissues were harvested under sterile conditions from the flexor digitorum profundus (FDP) tendon of hindpaws of adult New Zealand white rabbits. The harvested tendons were immediately placed in a 50 ml tube containing 20 ml PBS along with 100 U/ml penicillin and 100 U/ml streptomycin. Then they were minced into small pieces about 1 × 1 × 1 mm^3 and washed three times with PBS. The tissue fragments were digested with 0.15% collagenase NB4 (SERVA Electrophoresis, Germany) in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, N.Y.) for 2 h at 37 °C. The mixture was filtered to remove tissue residues through a sterile nylon mesh (Tetko, Elmsford, NJ), and the filtrate was centrifuged at about 200 g for 5 min. The supernatant was discarded and the cell pellet was resuspended in DMEM containing 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA), penicillin (100 U/ml), L-glutamine (292 μg/ml), streptomycin (100 U/ml), and ascorbic acid (50 μg/ml). Cells were then seeded onto 10-cm culture dishes and incubated in an incubator containing 5% CO_2 at 37 °C for different time points. The medium was changed every 2–3 days, and the cells were subcultured when reaching 80% confluence. Then, tenocytes were collected by 0.25% trypsin digestion for the use in the following procedures. For dermal fibroblast isolation and culture, rabbit skin was first treated with 0.1% dispase II (Roche, USA) in DMEM overnight at 4 °C to separate dermis from epidermis. Then the minced dermis was treated with 0.15% collagenase NB4 in DMEM at 37 °C on a shaker for 2 h. After centrifugation, the cell pellet was collected and cultured with the same method of tenocyte culture. The electrospun membrane was cut into discs of 15 mm diameter and placed into a 24-well plate. The membranes were immersed in 75% ethanol about 1 h for sterilization and then washed three times with PBS to remove residual ethanol. A suspension of cells was seeded onto the fibrous membrane surfaces and incubated in an incubator containing 5% CO_2 at 37 °C for different time points. The tissue culture plates were used as control.

2.2.2. Cell viability assay

Cells were seeded into 24 well plates at a density of 5 × 10^3 cells/cm^2. After incubation for 1, 3 and 5 days, AlamarBlue® (AbD Serotec, U.S.) was mixed with culture medium at 10 v% and incubated with cells for 4 h. 200 μl of the solution was then transferred into a 96-well plate and absorbance at 570 nm was measured using the spectrophotometer (Synergy 2, BioTek). The percentage reduction of AlamarBlue® was then converted according to the manufacturer's instruction.

2.2.3. Fluorescent staining and observation

Cells were seeded into 24 well plates at a density of 2 × 10^4 cells/cm^2. After incubation for 24 h, cell attachment was assessed by actin staining. Firstly, samples with cells were washed three times with PBS and then fixed in 4% paraformaldehyde for 10 min. Secondly, cells were washed repeatedly with PBS after removing the fixative, and permeabilized with 0.1% Triton X-100 (Sigma, USA) for 10 min. Then, samples with cells were stained with 20 μg/ml of phalloidin (Cytoskeleton, U.S.) for 30 min at room temperature according to instructions of the manufacturer. Finally, cells were stained with 1 μg/ml DAPI (Sigma, USA) for 5 min at room temperature before imaging using a confocal laser scanning microscope (Leica TCSSP2, Heidelberg, Germany). The average cell area on different surfaces was evaluated.

2.2.4. Cell proliferation assay

The proliferation of cells cultured on different samples was analyzed after 4 days of incubation by cell counting. The cells were collected from the surfaces of each specimen using 0.25% trypsin digestion and counted with a COUNTESS™ automated cell counter (Invitrogen, USA). The average number of cells adhered/cm^2 of surface was recorded. After fixation with 2.5% glutaraldehyde (Sigma, USA) and dehydration in a graded series of ethanol, cells growing on different samples were observed by SEM.

2.3. Preliminary animal study

All protocols of the animals were approved by the institutional review committee of Shanghai Jiao Tong University, School of Medicine. 72 adult New Zealand