Therapeutic strategies for flexor tendon healing by nanoparticle-mediated co-delivery of bFGF and VEGFA genes

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ABSTRACT

Tendon injuries are a common injury of musculocutaneous system. Due to the lack of sufficient cellularity and low growth factor activity, healing of disrupted digital flexor tendon is troublesome and the process is lengthy and ineffective. bFGF and VEGFA gene were proved to be responsible and critical for promoting tendon healing. How to continuously enhance expression of these genes is a challenge. In this study, we developed a combination therapeutic approach that corrects the fundamental problem underlying intrasynovial tendon healing with introduction of growth factor genes *via* non-viral vector nanoparticle. PLGA nanoparticles as vehicle were used to delivery bFGF+VEGFA genes into injured tendon tissues. The expression of bFGF and VEGFA was upregulated in the tenocytes after transfection. We injected nanoparticle/bFGF+VEGFA gene complexes into injured tendons producing sufficient amounts of these factors required during early tendon healing period. After treatment, the ultimate strength of repaired tendons treated with nanoparticle/bFGF+VEGFA plasmid complexes was significantly increased, and combination therapy could also enhance flexor tendon gliding function. Therefore, combination gene therapy *via* nanoparticles may be an effective biological strategy for tendon repair.

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1. Introduction

Lacking of sufficient cellularity and low growth factor activity, healing of disrupted digital flexor tendon is troublesome and the healing process of injured tendon is usually lengthy and the healing strength is quite weak in its early healing period [1,2]. Despite after a well-established and highly standardized surgical procedure for injured tendons, the strength of tendon healing in its early period is still limited, even facing the risk of rupture [3,4]. Many researchers and surgeons made efforts to seek methods to enhance the strength of tendons, such as suture technique and cells or drug therapy [5–9]. In recent years, molecular modulation has been a subject of intense interest, with reports of experimental success following *in vitro* and *in vivo* studies [10–12].

In tendon healing process, tenocytes proliferate, differentiate, synthesize and excrete extracellular matrix such as collagen and fibronectin with the effect of growth factors [2,13,14]. Basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGFA), the most potent stimulators that have been known, play

an important role in tissue healing and regeneration [15,16]. Our research group had proved that delivery of bFGF and VEGFA genes through a viral vector system are able to correct the insufficiency of the intrinsic healing capacity and lead to promoting tendon healing respectively [10,17], while the treatment effect of combination of bFGF and VEGFA is still unclear.

Gene therapy may continuously modulate target gene expression during therapeutic process. Due to the targeting property, gene therapy attracts increasingly more attention [18,19]. Gene delivery vectors determine the effect of gene therapy[20,21]. While viral vectors possess highly effective transfection, the side-effects involved toxicity, cellular immune response, oncogenicity and quality control cannot be ignored [22–24]. Therefore, non-viral vectors tend to be a superior choice because of their high biocompatibility and security [25,26].

In recent years, the application of nanoparticles as carriers for the treatments of a wide range of diseases has become a popular strategy with the advantages of biodegradability and controlled release ability [27–29]. Nanoparticles can be loaded with nucleic acids and easily penetrate the cell membrane, as a result of their small sizes less than 100 nm [30,31]. Our group previously found that PLGA nanoparticles have higher transfection efficiency for tenocytes [32].

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In this study, we attempted to promote the simultaneous expression of bFGF and VEGFA genes, in order to further enhance the strength in digital flexor tendons. Firstly, bFGF and VEGFA genes were constructed into pEGFP-N1 vectors respectively, and then the mixed generated plasmids were loaded into PLGA nanoparticles. Then the characteristics of the nanoparticle/plasmid complexes and transfection efficiency were evaluated. Finally, we injected the nanoparticle/plasmid complexes contained bFGF plus VEGFA into injured tendons to detect the treatment effect, comparing with single gene therapy and no treatment.

2. Material and methods

2.1. Preparation of expression vectors

According to genetic sequences of bFGF (Gene Bank accession: NM_002006) and VEGFA (NM_001171626), we constructed their open reading frames (ORF) into pEGFP-N1 vectors (GeneChem, China) to form overexpressed bFGF and VEGFA plasmids (pEGFP-bFGF and pEGFP-VEGFA). The constructed plasmids were sequenced to verify correct insertions of the bFGF and VEGFA ORF. The pEGFP-N1 vector harbors enhanced green fluorescence protein (EGFP) gene for using to detect transfection efficiency against tenocytes *in vitro* and *in vivo*.

2.2. Preparation of PLGA nanoparticles

Nanoparticles were obtained by double emulsion method as previous study [33–36]. Firstly, 200 mg Poly(D,L-lactide-co-glycolide) (PLGA, lactide:glycolide (65:35)) was fully dissolved in 2 mL dichloromethane (DCM), and then emulsified in 6 mL of a 7% (w/v) poly(vinylalcohol) (PVA) (Sigma Aldrich St. Louis, MO, USA) aqueous solution by sonication using a Sonoplus HD 2070 ultrasonic homogenator (Bandelin electronic, Berlin, Germany) for 60 s to produce the primary emulsion. Subsequently, the above primary emulsion was added into 100 mL of a 1% (w/v) PVA aqueous solution, and sonicated again for 180 s to form the double emulsion, which was stirred gently for 24 h at room temperature for evaporating the residual dichloromethane. The PLGA nanoparticles were isolated by centrifugation (13,000 rpm, 5 min) at 4 °C and washed twice with deionized water. Finally, the nanoparticles were suspended in the deionized water and dispersed by sonication.

2.3. Preparation of nanoparticle/plasmid complexes

In order to load the plasmids onto the nanoparticles, the nanoparticles were firstly modified with polyethyleneimine (PEI) to make them positively charged for attracting plasmids with negatively charged. Briefly, 100 μL of PLGA nanoparticle solution (10 μg/μL) was mixed with 200 μg PEI (100 μg/μL) in deionized water, and then the mixture solution was added to the plasmid solutions at N/P ratio of 6:1 (nitrogen of PEI to phosphate of plasmid) ratios and vortexed gently and incubated for 20 min at room temperature to formulate PLGA nanoparticle/plasmid complexes.

2.4. The characterization of nanoparticle/plasmid complexes

Dynamic light scattering (DLS) method was used to determine the average diameter and size distribution of PLGA nanoparticles/plasmid complexes by using a Brookheaven BI9000AT system (Brookheaven Instruments Corporation, Austin, Texas, USA). The size and morphology of the complexes were characterized by using the scanning electron microscopy (SEM, Hitachi, S-3400N, Tokyo, Japan). Briefly, the PLGA nanoparticles/plasmid complexes were coated with platinum after freeze-drying, then photographed under an SEM.

2.5. Culture of primary tenocytes

After washing and sterilizing, the feet of white Leghorn chickens were cut down and the long flexor digitorum profundus (FDP) tendon segments were harvested. The tendon segments were then cut into 1–2 cm pieces with ophthalmic scissors and placed on the culture dish for explant culture in complete medium which contained 90% DMEM (Dulbecco's modified Eagle's medium), 10% FBS, 100 mg/mL streptomycin, 100 U/mL penicillin. The culture dishes were placed in incubator at 37 °C in a humidified atmosphere of 5% CO_2. About 10–14 days, the cells would migrate from tissue explants and proliferate rapidly to form mono-layer confluence. At that time, tissue explants were removed and the tenocytes on the dishes were digested and passaged for following experiments.

2.6. Cell viability

Cell viability was detected by using the MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay. Briefly, the tenocytes were seeded onto a 96-well plate with an initial density of 0.5×10^4 cells/well in 0.1 mL of culture medium. After 24 h, 100 μL of MTT dye (0.5 mg/mL, Sigma) was added to each well and cultured for another 4 h. Then the supernatant was removed, 150 μL of dimethyl sulphoxide (DMSO) (Sigma, St. Louis, MO, USA) was added to each well containing cells. The absorbances of the plate were measured at 570 nm. Untreated cells were taken as control with 100% viability. All experiments were performed in triplicates.

2.7. In vitro transfection efficiency

Tenocytes were seeded on a 6-well plate at an initial density of 1×10^5 cells/well in 2 mL of culture medium and incubated until the coverage ups to about 70–80%. Transient transfections were conducted using pEGFP-N1 negative plasmids(Neg), liposome/pEGFP-N1 negative plasmid complexes(Lipo-Neg), nanoparticle/pEGFP-N1 negative plasmid complexes (NP-Neg), nanoparticle/pEGFP-bFGF complexes (NP-bFGF), nanoparticle/pEGFP-VEGFA complexes (NP-VEGFA) and nanoparticle/pEGFP-bFGF+pEGFP-VEGFA complexes (NP-bFGF+VEGFA) containing 4 ug plasmids in different wells. These complexes were added to the cell culture medium and incubated for 24 h, 48 h and 72 h respectively. Then the medium was replaced by fresh complete medium. After washing 3 times with 1 mL PBS, the cells were observed with the fluorescence microscopy (Leica DMR 3000; Leica Microsystem, Bensheim, Germany) in FITC channels (excitation 488 nm, emission 518 nm) at different time points. In order to quantify transfection efficiency, cells in different groups were digested and resuspended for flow cytometry analysis by FACS Calibur flow cytometer (BD Bioscience, San Jose, CA, USA).

2.8. Animal surgical procedure and delivery of plasmid to injured tendons *in vivo*

Because of the similarity of flexor mechanism between chickens and human, we chose long toes of the white Leghorn chickens aged one year as the experimental model. One hundred and ninety-six long toes in both feet of 98 chickens were used in this study. The chickens were randomly divided into four time-points and five groups, nanoparticle/pEGFP-bFGF+pEGFP-VEGFA complexes group (NP-bFGFP+VEGFA), nanoparticle/pEGFP-bFGF complexes group (NP-bFGF), nanoparticle/pEGFP-VEGFA complexes group (NP-VEGFA), nanoparticle/pEGF-N1 negative plasmid complexes group (NP-Neg) and no treatment group (Control) at postoperative weeks 1, 2, 4 and 6. We anesthetized the chickens by intramuscular injection with 10% chloral hydrate and applied Elastic bandages