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The effect of mechanical stimulation on the maturation of TDSCs-poly(L-lactide-co-e-caprolactone)/collagen scaffold constructs for tendon tissue engineering

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ABSTRACT

Mechanical stimulation plays an important role in the development and remodeling of tendons. Tendon-derived stem cells (TDSCs) are an attractive cell source for tendon injury and tendon tissue engineering. However, these cells have not yet been fully explored for tendon tissue engineering application, and there is also lack of understanding to the effect of mechanical stimulation on the maturation of TDSCs-scaffold construct for tendon tissue engineering. In this study, we assessed the efficacy of TDSCs in a poly(L-lactide-co-ε-caprolactone)/collagen (P(LLA-CL)/Col) scaffold under mechanical stimulation for tendon tissue engineering both *in vitro* and *in vivo*, and evaluated the utility of the transplanted TDSCs-scaffold construct to promote rabbit patellar tendon defect regeneration. TDSCs displayed good proliferation and positive expressed tendon-related extracellular matrix (ECM) genes and proteins under mechanical stimulation *in vitro*. After implanting into the nude mice, the fluorescence imaging indicated that TDSCs had long-term survival, and the macroscopic evaluation, histology and immunohistochemistry examinations showed high-quality neo-tendon formation under mechanical stimulation *in vivo*. Furthermore, the histology, immunohistochemistry, collagen content assay and biomechanical testing data indicated that dynamically cultured TDSCs-scaffold construct could significantly contributed to tendon regeneration in a rabbit patellar tendon window defect model. TDSCs have significant potential to be used as seeded cells in the development of tissue-engineered tendons, which can be successfully fabricated through seeding of TDSCs in a P(LLA-CL)/Col scaffold followed by mechanical stimulation.

1. Introduction

Tendons have a poor self-regenerative capacity due to their low cellularity and vascularity. Tendon regeneration after injury remains a formidable challenge [1]. Developments in tissue engineering may provide a promising alternative therapy for tendon injuries [2].

A long-standing focuses in tissue engineering is to recover the damaged tissues with full regeneration of their biological functions. Three elements are necessary to develop functional tissue-engineered tendons with good biological activity and mechanical properties: (1) seeded cells which can retain good proliferation and differentiation toward tenogenic lineage; (2) a biological scaffold which can provide a three dimensional (3D) space enough for cell growth; and (3) proper mechanical or chemical stimulation [3]. In recent years, studies on tendon tissue engineering have entered into a phase of clinical research [4,5]. However, difficulty in finding appropriate seeded cells hinders the further development of tendon tissue engineering [6]. Tendon-derived stem cells (TDSCs) are a new type of cells and have been isolated successfully from human adults [7], rats [1], mice [8] and rabbits [9]. TDSCs have general characteristics of stem cells, such as colony formation, self-duplication, multi-directional differentiation potential, etc [1]. TDSCs are superior to mesenchymal stem cells (MSCs) from the same individual source in the aspect of multi-directional differentiation and self-renewal capacities, and TDSCs can express more tenogenic differentiation-related mRNA (such as, tenomodulin, scleraxis, type I collagen (Col I), decorin and biglycan) than MSCs

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[10]. The fibrin glue construct with TDSCs can repair the injured tendons earlier and preferable than that without TDSCs [11], thus TDSCs can be used as seeded cells for tendon regeneration. At present, there are few studies to focus on the utilization of TDSCs as seeded cells in tendon tissue engineering or to explore the vast potential of TDSCs for tendon regeneration.

Mechanical microenvironment can affect cell proliferation, migration, differentiation and apoptosis, as well as tissue development [12]. Tendons are subjected to a continuous action of mechanical load (mainly tensile load) generated by muscle contraction in the body [13]. The growth, development and regeneration of tendons are closely related with mechanical stimulation [14]. Low-intensity mechanical stimulation promotes the proliferation and tenogenic differentiation of TDSCs isolated from rabbit patellar tendons, while high-intensity mechanical stimulation induces the adipogenic, osteogenic, and chondrogenic differentiation of TDSCs [15]. It has been found that the collagen synthesis capacity of TDSCs isolated from mice after 1-week treadmill exercise is increased significantly [16]. Therefore, it is feasible to induce the tenogenic differentiation of TDSCs with mechanical stimulation. Different roles of mechanical stimuli in the developing of tissue-engineered tendons have been widely studied [5,17,18]. Mechanical stimulation significantly increased cell proliferation over the 14-day culture period by at least eightfold in the tissue-engineered tendons [19]. In addition, mechanical stimulation contributed to the formation of collagen fibers and the production of extracellular matrix (ECM) along the loading direction [20]. And mechanical stimulation can improve or optimize the mechanical properties of tissue-engineered tendons, including rigidity, Young's modulus, and maximum tensile stress [21–23]. Therefore, mechanical stimulation undoubtedly plays an important role in successfully developing tissue-engineered tendons.

In the preliminary studies, we investigated the utilization of 3D aligned poly(L-lactide-co-ε-caprolactone)/collagen (P(LLA-CL)/Col) scaffolds in tendon tissue engineering. The results have shown that P(LLA-CL)/Col scaffolds provide a positive environment for cell adhesion, alignment and infiltration, and also provide desirable mechanical properties for tissue-engineered tendons [24]. Aligned nanofibers provide an instructive microenvironment for the tenogenic differentiation of TDSCs than random nanofibers [1]. Meanwhile, mechanical stimulation leads to significant changes in the fibroblastic differentiation potential of MSCs [25]. However, the tenogenic differentiation potential of TDSCs cultured on the aligned scaffold under mechanical stimulation was not studied extensively. Also, the effects of the use of dynamically cultured TDSCs-scaffold construct for repairing the injured tendons have not been studied.

This study aimed to investigate the maturation of tissue-engineered tendons formed by TDSCs and 3D aligned P(LLA-CL)/Col under mechanical stimulation *in vitro* and *in vivo*, and the effects of dynamically cultured tissue-engineered tendons in promoting the regeneration of injured rabbit patellar tendons.

2. Materials and methods

2.1. Scaffold fabrication

P(LLA-CL)/Col scaffolds were fabricated using a dynamic water flow system by electrospinning based on our preliminary studies [24]. Briefly, the dynamic water flow system was composed by a basin with an 8 mm hole on its bottom to produce a vertex flow and a pump to recycle water drained into the tank below the basin. P(LLA-CL) (LA:CL = 50:50, Mw = 300,000, Nara Medical University, Japan) and Type I collagen (Col I) (Ming-Rang Co. Ltd. Chengdu, China) were dissolved into 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP, Da-Rui Co. Ltd. Shanghai, China) to give a blended solution (8 w/v %) of 90:10, then the blended solution was sprayed at 1.0 mL/h under a high voltage of 15 kV, 15 cm above the vertex flow. After HFIP was evaporated, electrospun nanofibers were formed and stacked on the water surface, then they were twisted into yarns under the action of vertex flow, and collected by a rotating mandrel (60 r/min) to form P(LLA-CL)/Col scaffolds. The obtained scaffolds (about 150 μm thick) were cryopreserved at −80 °C for 2 h, then freeze-dried overnight and preserved in a vacuum container. Macroscopic evaluation and scanning electron microscopy (SEM, Fig. 1B) showed the morphology coincident to our preliminary study results [24].

2.2. Cell isolation and culture

Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) of the Third Military Medical University. Primary TDSCs were harvested from the bilateral patellar tendons of Oryctolagus cuniculus, isolated according to the previously described procedures [9,26], and then resuspended in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (HyClone) medium containing 20% fetal bovine serum (FBS; Invitrogen) and 1% penicillin and streptomycin (HyClone). The resultant suspension was diluted to obtain a 1 cell/μl single-cell suspension which was dropped into a 96-well plate (Corning) and cultured for 8–10 days at 37 °C and 5% CO_2 till TDSCs clones were observed. Individual cell clones were isolated by the topical application of trypsin (Gbico) under the microscope (Olympus BX51). The isolated TDSCs clones were collected and transferred with a micro-pipette into a T25 culture flask (Corning) for further culture. The culture medium was replaced in every three days, and the cells were digested with 0.25% trypsin and passaged after reaching 90% confluence.

2.3. In vitro study

2.3.1. Static and dynamic culture of TDSCs-engineered tendons

The scaffold samples (length × width: 4.5 × 2.5 cm) were placed in tissue-culture polystyrene plates (TCPSs, Costar) and sterilized with 70% ethanol for 30 min, then rinsed with sterile phosphate-buffered saline (PBS), and subsequently immersed in DMEM/F12 medium overnight. TDSCs (passage 3, Fig. 1A) were seeded on the scaffolds (1×10^5 cells/scaffold). The cell-seeded scaffolds were cultured in an incubator (37 °C, 5% CO^2) for 4 h to promote cell adhesion. Then, the culture medium was added into the wells.

After the cell-scaffold constructs (Fig. 1C) were subjected to static culture for 24 h, they were curled into concentric 3D constructs along their 4.5 cm long axis, and then fixed on the two opposing tissue fixing columns of the culture chamber by the nylon threads (Fig. 1D, green arrows (in web version)) under sterile conditions. There were totally 3 cell-scaffold constructs/culture chamber. About 80 ml culture medium was added into each culture chamber. The mechanical stretch was performed with a mechanical traction stimulation system developed in our preliminary studies [27]. For the dynamic mechanical stimulation group, the cell-scaffold constructs were placed into a chamber, and then stretched at 4% elongation in length and 0.5 Hz [15], 2 h per day for a total of 14 days. The cell-scaffold constructs in the static group was cultured statically within another chamber under the same conditions.

2.3.2. Cell viability and morphology

The viability and morphology of cells were evaluated with Live/Dead stain (Invitrogen) according to the manufacturer's instructions [28]. The stained samples were photographed at an excitation wavelength of 488/594 nm under laser confocal microscope (LSM 510, Zeiss, Germany). The number of viable cells and the total number of cells were counted from the images by imageJ software (ImageJ 1.46 r; NIH), and then their ratio was calculated ($n = 3$).

2.3.3. Cell proliferation

The content of total DNA was measured by PicoGreen dsDNA assay (Invitrogen) [25]. The harvested samples were homogenized by Triton-X (Sigma) and ultrasound. The fluorescence was detected at an excitation/emission wavelength of 485/535 nm using a microplate reader (Model 550; Bio-Rad, USA). The standard curve was plotted with DNA concentration-dependent fluorescence intensity, and was used to calculate the number of cells in the samples.

2.3.4. Histological analysis

The cell-scaffold constructs were harvested and then immediately fixed in 4% neutral formalin, gradient-dehydrated with alcohol and embedded into paraffin. The longitudinal sections (5 μm) were stained with hematoxylin and eosin to evaluate cell growth and infiltration.

2.3.5. Real-time polymerase chain reaction (PCR) analysis

In order to clarify the effects of mechanical stimulation on the differentiation of TDSCs, the levels of Col I, type III collagen (Col III), decorin, tenascin C, biglycan (tenogenic differentiation-related genes), Runx2 (osteogenic gene), and type II Collagen (Col II), aggrecan (chondrogenic genes), were detected in dynamic group and static group after 7 and 14 days of culture, respectively. TDSCs collected immediately prior to scaffold seeding served as controls. Total RNA was extracted with TRIzol reagent (Invitrogen) according to the instructions. 500 ng RNA was put into a 20 μL reaction system for reverse transcription with PrimeScript™ RT reagent kit with gDNA eraser (RR047A, Takara) according to the manufacturer's instructions. Real-time PCR was performed using 2× SYBR® Green PCR Master Mix (Applied Biosystems) on a Real-Time PCR System (Applied Biosystems 7500). All primer sequences (Sangon Biotech Co., Ltd., China) were designed using primer 5.0 software and summarized in Table 1. Each sample was tested for three times and 3 PCR cycles