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Engineering of extensor tendon complex by an *ex vivo* approach

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ABSTRACT

Engineering of extensor tendon complex remains an unexplored area in tendon engineering research. In addition, less is known about the mechanism of mechanical loading in human tendon development and maturation. In the current study, an *ex vivo* approach was developed to investigate these issues. Human fetal extensor tenocytes were isolated, expanded and seeded on polyglycolic acid (PGA) fibers that formed a scaffold with a shape mimicking human extensor tendon complex. After *in vitro* culture for 6 weeks, 7 cell-scaffold constructs were further *in vitro* cultured with dynamic mechanical loading for another 6 weeks in a bioreactor. The other 14 constructs were *in vivo* implanted subcutaneously to nude mice for another 14 weeks. Seven of them were implanted without loading, whereas the other 7 were sutured to mouse fascia and animal movement provided a natural dynamic loading *in vivo*. The results demonstrated that human fetal cells could form an extensor tendon complex structure *in vitro* and become further matured *in vivo* by mechanical stimulation. In contrast to *in vitro* loaded and *in vivo* non-loaded tendons, *in vivo* loaded tendons exhibited bigger tissue volume, better aligned collagen fibers, more mature collagen fibril structure with D-band periodicity, and stronger mechanical properties. These findings indicate that an extensor tendon complex like structure is possible to generate by an *ex vivo* approach and *in vivo* mechanical loading might be an optimal niche for engineering functional extensor tendon.

1. Introduction

Although the lack of an appropriate tissue source remains a problem for tendon reconstruction, the regeneration of tendon through the use of autologous tendon cells combined with scaffolds is now feasible [1]. Previously, studies have demonstrated that functional tendon tissue could be generated *in vivo* and be used to repair tendon defect in hen [2], pig [3], rabbit [4–9] and other models [10].

However, it would be optimal to provide patients a relatively mature tendon graft for implantation instead of a cell-scaffold construct. Previously, we have shown that a preliminary tendon structure could be generated by *in vitro* culture under static tension [11]. Nevertheless, it remains unclear whether *in vitro* engineered tendon would become more mature after *in vivo* implantation, particularly under mechanical loading, an important niche for tendon development after birth.

According to the literature, tendon tissue engineering research has mainly focused on the construction of flexor tendon structure or a linear tendon structure [2–9]. Different from flexor tendon, extensor tendon at zones III and IV of human hand is a complex structure that performs important hand functions [12]. However, no attempt has been made to engineer an extensor tendon complex that may potentially be applied for hand function reconstruction. Considering the importance of extensor tendon in hand function and the importance to define a proper niche for development of this complex, we employed human fetal extensor tenocytes to explore the possibility of generating extensor tendon complex *in vitro* and to investigate the role of *in vivo* mechanical loading in extensor tendon development and maturation.

2. Materials and methods

2.1. Animals

Fourteen nude mice (7 males and 7 females, 8 weeks old) were involved in this study and the animals were purchased from SLAC National Rodent Laboratory Animal Resources (Shanghai, China). An institutional review committee of Shanghai Jiao Tong University School of Medicine approved all animal study protocols.

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2.2. Harvesting and culture of human extensor tenocytes

Protocols for the handling of human tissue and cells were approved by the Ethics Committee of Shanghai Jiao Tong University School of Medicine and aborted fetuses were donated by the patients for research purpose. Briefly, extensor tendons were harvested from the hand dorsal area of 3-month old aborted fetuses and adjacent tissues were stripped. After thorough wash in phosphate-buffered saline (PBS) and soaking in 2.5% chloramphenicol solution for 10 min, the tendons were washed again 3 times in PBS and then were cut into small fragments followed by enzyme digestion with 0.2% collagenase II (Worthington, Freehold, NJ) in serum-free Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, NY) at 37 °C on a rotator as previously described [2,3]. The resulting cell suspension harvested at 4 h post-digestion was filtered through a sterile nylon mesh (Tetko, Elmsford, N.J.) to remove tissue residues. The filtrate was further centrifuged and cell pellets were washed twice with PBS and then resuspended in DMEM culture medium containing 10% fetal bovine serum (FBS, Gibco), L-glutamine (292 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), and ascorbic acid (50 μg/ml). The extracted cells were plated on 100-mm culture dishes ($5 \times 10^5/cm^2$) and incubated at 37 °C in a humidified atmosphere containing 95% air and 5% carbon dioxide with medium change every 2–3 days. When cultured primary cells reached 80% confluence, they were detached by the treatment of 0.25% trypsin and 0.1% ethylenediaminetetraacetic acid (EDTA, Gibco) and subcultured at the same density ($5 \times 10^5/cm^2$). Cultured cells before passage 3 were used for experiments.

2.3. Preparation of scaffold material

Long polyglycolic acid (PGA) fibers with a diameter about 20–30 μm were provided by Shanghai Ju Rui Biomaterials Company Inc (Shanghai, China). These fibers were arranged into a shape of extensor tendon complex and secured on a custom-made spring formed with stainless steel. As shown in Fig. 1, 50 mg PGA long fibers were used for the central slip to form a cord 5 cm long and 0.5 cm wide, and 35 mg PGA long fibers were used for each lateral band with a length of 6 cm and a width of 0.3 cm. An angle of about 20–25° was formed between the central slip and the lateral bands.

The formed scaffold complex was first soaked in Povidone Iodine for 10 min, washed with 75% ethanol and soaked in 75% ethanol for 60 min. After 3 washes in PBS, the scaffold complex was pre-incubated in DMEM supplemented with 10% FBS at 37 °C overnight in an incubator.

2.4. In vitro *engineering of extensor tendon*

In vitro cultured tenocytes were harvested with trypsin–EDTA treatment, centrifuged and resuspended in culture medium at a concentration of 2×10^7/ml. After removal of culture medium by suction, the scaffold complexes were evenly seeded with suspended cells (0.5 ml per construct). The cell-scaffold constructs were kept in an incubator at 37 °C for 4 h to allow for complete adhesion of the cells to the scaffold and then culture medium was added followed by 2 weeks of static culture with medium change every other day. Afterwards, they were removed from the spring and transferred to a custom-made bioreactor that was able to provide a dynamic stretch to the constructs with a frequency of 6 times per minute, each lasting for 5 s. The mechanical stretch was loaded for 60 min per day for total 4 weeks or 10 weeks with stretch amplitude of one-tenth length of the engineered tendons.

2.5. Experimental design

Totally 21 cell-scaffold constructs were involved in this study. Among them, 7 constructs were for *in vitro* tendon engineering by 2 weeks of static culture followed by 10 weeks of dynamic culture. The rest of 14 constructs were *in vitro* cultured for 2 weeks statically and 4 weeks dynamically and then *in vivo* implanted for another 14 weeks. Among 14 *in vivo* implanted constructs, 7 were subjected to mechanical loading whereas the other 7 were not loaded. As a control, 6 scaffold complexes without cells were *in vitro* cultured for total 12 weeks on the spring.

2.6. In vivo *remodeling of* in vitro *engineered tendons*

In order to determine the effect of *in vivo* mechanical loading on tissue development and maturation, a nude mouse model was applied. Briefly, 14 mice were randomly divided into two groups with equal number of females and males. After anesthetization with intraperitoneal injection of pentobarbital sodium (20 mg/kg), an incision was made on the dorsum and a subcutaneous pocket was created to expose fascia muscularis of limbs on both sides, fascia nuchae on dorsonuchal area and fascia of musculi sacrospinalis on sacral area. To provide *in vivo* mechanical loading, the engineered central slip was sutured to fascia nuchae at the top and to fascia of musculi sacrospinalis at the bottom, whereas the four ends of engineered lateral bands were sutured to fascia muscularis of limbs, respectively, with Ethilon 6-0 suture (Johnson & Johnson, USA) (Fig. 3G). Therefore, natural movement of mouse limb or body could provide mechanical loading on various parts of the sutured tendons. As a control of no loading, the engineered tendon was simply implanted in the subcutaneous pocket without suturing (Fig. 3D). At the end of 14 week implantation with animal euthanasia, the implanted tissues were harvested for various examinations.

2.7. Gross and histological examinations

All harvested specimens were measured for their width and thickness at the central area where the central slip and the lateral bands joined, and observed for their color, vascularization and fibrotic adhesion to surrounding tissue. In each group, one entire engineered complex tissue and partial tissue from other complexes were randomly selected and fixed with 4% paraformaldehyde, paraffin embedded and sectioned to 5 μm thickness for hematoxylin and eosin staining to examine tissue structure particularly for cell density, collagen formation and alignment. In addition, polarized microscopic examination was applied to evaluate collagen structure and maturation level as well as PGA degradation.

2.8. Scanning electron microscopic (SEM) examination

After 7 days of *in vitro* culture, part of the cell-scaffold construct was prepared for SEM examination. Briefly, samples were prefixed with 2% glutaraldehyde for 2 h at 4 °C and washed twice with PBS at 4 °C followed by post-fixation with 1% osmic acid for 2 h at 4 °C. After 2 washes in PBS, the samples were dehydrated with gradient ethanol and dried to a critical point (HCP-2, Hitachi, Japan). The samples were then mounted, sputter-coated with gold (BAL-TEC, Philips, Eindhoven, Netherlands), and examined with a scanning electron microscope (Philips-XL-30) to observe cell attachment and matrix production on PGA scaffold.

2.9. Transmission electron microscopic (TEM) examination

Tissue samples were randomly selected from the harvested engineered tendons of all the 3 groups and were prepared similar to SEM. After dehydration with gradient ethanol, the specimens were further treated with 100% propylene oxide twice, each for 15 min, followed by embedding for 12–18 h with a mixture of propylene oxide and embedding resin (Embed 812, Electron Microscopy Sciences, Fort Washington, PA) in 1:1 ratio, and finally were polymerized in an oven at 60 °C overnight. The prepared samples were sectioned by a diamond knife (Leica Ultracut R) to 70–90 nm thickness, followed by staining with uranyl acetate and lead citrate for image contrast. All the specimens were viewed by a transmission electron microscope (Philips CM 120) to examine the distribution and to measure the diameter of collagen fibrils in the engineered tendons. KS400 Image Analysis Software (version 3.0, ZEISS) was applied to analyze the cross-sectional image of the collagen

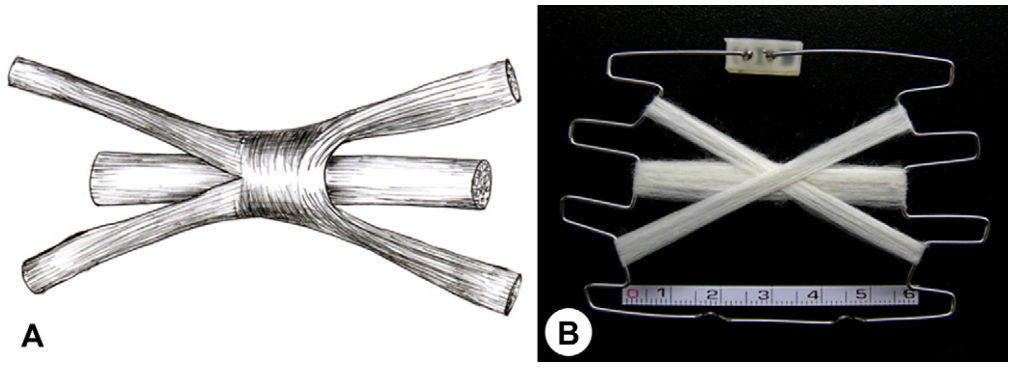

Fig. 1. Design of extensor tendon complex scaffold. (A) Schema chart showing the central slip and two lateral bands. (B) PGA scaffold secured on a custom-made spring to mimic the complex structure.