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The roles of TGF-β1 gene transfer on collagen formation during Achilles tendon healing

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ABSTRACT

Collagen content and cross-linking are believed to be major determinants of tendon structural integrity and function. The current study aimed to investigate the effects of transforming growth factor (TGF)-β1 on the collagen content and cross-linking of Achilles tendons, and on the histological and biomechanical changes occurring during Achilles tendon healing in rabbits. Bone marrow-derived mesenchymal stem cells (BMSCs) transfected with the TGF-β1 gene were surgically implanted into experimentally injured Achilles tendons. Collagen proteins were identified by immunohistochemical staining and fiber bundle accumulation was revealed by Sirius red staining. Achilles tendons treated with TGF-β1-transfected BMSCs showed higher concentrations of collagen I protein, more rapid matrix remodeling, and larger fiber bundles. Thus TGF-β1 can promote mechanical strength in healing Achilles tendons by regulating collagen synthesis, cross-link formation, and matrix remodeling.

Introduction

The restoration of mechanical strength is the main goal of tendon healing. Of the many substances comprising the matrix of the tendons, collagen fibers are largely responsible for their mechanical strength [1,2]. Previous reports have shown that several growth factors are functionally important for tendon healing [3], and transforming growth factor (TGF)-β1 has been shown to be able to stimulate collagen production.

The biosynthetic pathway responsible for collagen production is very complex. All types of collagen are produced initially as a larger precursor molecule called procollagen [4]. In a previous study, rat Achilles tendons were transected and immediately repaired, and two different doses of TGF-β1 dissolved in phosphate-buffered saline were injected locally at the repair site. Dose-dependent increases in the expression of procollagen type I and III mRNA were found after TGF-β1 administration [5]. In another study, three separate cell lines—sheath fibroblasts, epitenon and endotenon tenocytes—were isolated from rabbit flexor tendons and cultured separately. The addition of TGF-β1 to the cell culture media resulted in a significant increase in the production of collagen I and III proteins [6].

The cross-linking process is the critical step that gives the collagen fibers their strength [4]. This step is promoted by lysyl oxidase, the synthesis of which can be up-regulated by TGF-β1. A previous study reported that TGF-β1 significantly increased steady state lysyl oxidase mRNA levels, lysyl oxidase proenzyme, and its extracellular proteolytic processing in MC3T3-E1 osteoblastic cells [7]. A later study demonstrated that lysyl oxidase bound directly to mature TGF-β1. Furthermore, it has been demonstrated that lysyl oxidase suppressed TGF-β1 signaling via its amine oxidase activity, and that this suppression was rescued by inactive lysyl oxidase [7,8].

Matrix remodeling is also an important part of tendon healing. During the healing process, type III collagen appears transiently, and is then replaced by type I collagen [9]. The advantage of type III collagen is believed to be its ability to rapidly form cross-links and so stabilize the repairing site [10]. Nevertheless, an excess of type III collagen may cause a reduction in the tensile strength, since type III collagen fibers are thinner and more extensible than those of type I collagen [5,7,10]. TGF-β1 may participate in matrix remodeling by modulating collagen synthesis. A previous study indicated that TGF-β1 decreased the levels of procollagen type III mRNA, and increased the levels of procollagen type I mRNA in type I collagen-coated plates [11].

In the present study, we tested the hypothesis that TGF-β1 could promote the mechanical strength of healing Achilles tendons by regulating collagen synthesis, cross-link formation, and matrix

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remodeling in vivo. We used bone marrow-derived mesenchymal stem cells (BMSCs) as a platform for gene transfer, to allow persistent TGF-β1 gene expression.

Materials and methods

Vector construction. The human TGF-β1 cDNA was inserted in place of the E1 region in the shuttle plasmid pGEM-T-Easy (whose expression is driven by the cytomegalovirus promoter) to construct the pGEM-T Easy-TGF-β1 plasmids. The recombinant TGF-β1 virus was generated by Cre-Lox-driven recombination in CRE8 cells [12]. The recombinant virus was purified and amplified in 293A cells [12].

BMSC isolation and expansion. BMSCs were obtained from anesthetized rabbits and processed as previously described [13]. BMSCs were also obtained from the cavities of rabbit femurs and tibias, then seeded into 25 cm^2 flasks and cultured at a density of 1×10^6 cells/ml at 37 °C in a mixture of 95% air and 5% CO_2 in DMEM (GIBCO, USA) containing 10% fetal bovine serum (FBS) (GIBCO). A small number of cells developed into visible symmetric colonies by 6–8 days. Third-passage cells were used for the experiments.

Gene transfer. The recombined virus was added to culture flasks containing third passage BMSCs ($4.5 \times 10^{14}/10^6$ cells) and incubated in DMEM without 10% FBS for 4 h. The cells were isolated by trypsin and centrifugation 48 h after gene transfer.

Animal model and treatment. Male New Zealand White rabbits were used according to the guidelines of the Institutional Animal Care and Use Committee and with the approval of our institutional review board. The rabbits were 4.5 ± 1.2 months old and weighed 2 ± 0.5 kg (mean ± standard deviation). Forty-eight rabbits were used for the Achilles tendon healing experiments. Using aseptic techniques, a longitudinal skin incision was made directly over the Achilles tendon of one hind limb. The gastrocnemius tendon was dissected free from the plantaris and soleus tendons. A complete transverse hole (3-mm diameter) was made using a punch through the middle of the Achilles tendon [14]. The BMSCs were then added into and around the hole (see below for details). The paratenon tissue was then sutured using Prolene 6/0 sutures and the skin was closed using Prolene 4/0 suture. The procedure was then repeated on the other hind limb. Achilles tendons randomly received BMSCs with or without TGF-β1 genes. Achilles tendons that received BMSCs without TGF-β1 genes were assigned to Group 1, while tendons that received BMSCs with TGF-β1 genes were assigned to Group 2 (n = 12 per group at each time point). The rabbits were not immobilized postoperatively and were fed ad libitum.

Histological analyses. Rabbits were sacrificed at 1, 2, 4, and 8 weeks. Tendon specimens were embedded in paraffin and sectioned at 6 μm for Sirius red and hematoxylin and eosin staining. Sections used for Sirius red staining and immunohistochemistry were not stained with hematoxylin.

Immunohistochemistry. Monoclonal primary antibodies for type I collagen (1:400, ABCAM, US) and type III collagen (1:1000, Daiichi Fine Chemical Co, Japan) were used for staining. The streptavidin–biotin (Lab Vision; Zhongshanjinqiao Corporation, China) method, coupled with a diaminobenzidene chromogen system (Lab Vision; Zhongshanjinqiao Corporation) was used for visualization.

Sirius red staining. Sirius red (0.1 g; Sigma–Aldrich, St. Louis, MO, USA) was diluted in 1000 ml of 0.1% trinitrophenol. Sections were incubated in the solution at 37 °C for 2 h [15].

Biomechanical testing. Tendons used for biomechanical testing were harvested from the calcaneus and musculotendinous junction. Six specimens were harvested from each group at 1, 2, 4, and 8 weeks. The processing was performed as previously described: The two ends of the tendon were clamped securely to the clamps of a biomechanical testing machine (Model WDW3020; ShuaiHua, Changchu, China), 3 mm away from the edges of the repair site. Measurements of the repair site were made using vernier calipers. The specimen was mounted onto the biomechanical testing machine, and a preload force of 2 N was applied to provide initial tension. Once under tension, the tendons were then loaded to failure at a constant rate of 10 mm/min. Data collected were used to calculate the material and structural properties of each specimen [16].

Statistical analyses. The number of animals used in the study was decided on after consultation with our Institution's biostatistician.

A repeated-measures ANOVA with Group as the between-subject factor (Group 1 or Group 2) and Time as the within-subject factor (1 week, 2 weeks, 4 weeks, or 8 weeks) was conducted to investigate the effects of TGF-β1 gene transfer on the maximum load and elastic modulus, with the level of significance set at 0.05. Statistical analyses were performed using SPSS software (SPSS v12.0; SPSS).

Results

Hematoxylin and eosin staining

Hematoxylin- and eosin-stained sections of Achilles tendons after gene transfer are shown in Fig. 1.

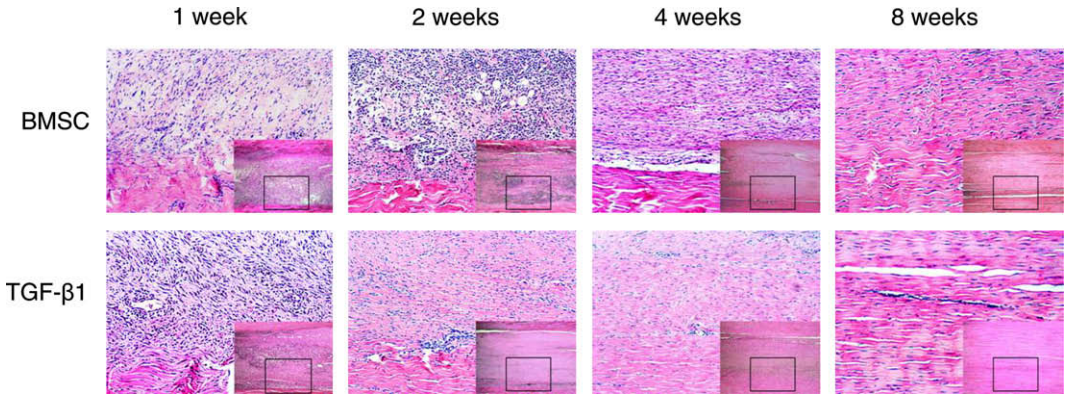

Fig. 1. Hematoxylin- and eosin-stained injured Achilles tendon after gene transfer. The images in the lower right corners were photographed at 40× magnification. The healing tissue is in the center of the picture, while the normal tissue is above and below the healing tissue. The main parts of the figures represent the areas in the black boxes, photographed at 200× magnification. In the main picture, healing tissue is in the upper part of the picture, and normal tissue is at the bottom. The Achilles tendons treated with BMSCs with TGF-β1 gene transfer showed more rapid tissue organization than those without TGF-β1, at all time points.