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Dexamethasone inhibits the differentiation of rat tendon stem cells into tenocytes by targeting the scleraxis gene

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ABSTRACT

Glucocorticoid-induced tendon rupture is very common in clinical practice, and the overall outcome of surgical suture repair is rather poor. The mechanism remains unclear, and effective treatments are still lacking. In the present study, we investigated the effect of dexamethasone on the differentiation of rat tendon stem cells (TSCs) to tenocytes and the underlying molecular mechanisms and found that dexamethasone inhibits the differentiation of TSCs to tenocytes by analyzing the development of long, spindle-shaped cells and detecting the expression of tenocyte markers type I collagen and tenomodulin (TNMD) at both the mRNA and protein levels. We also discovered that after treatment with dexamethasone, the scleraxis expression level is downregulated in vitro and in human specimen. Chromatin immunoprecipitation (ChIP)-PCR showed that dexamethasone promotes glucocorticoid receptor interacted with the TGGAAGCC sequence located between −734 and −726 base pairs (bp) upstream of the start codon of the scleraxis gene. Furthermore, TSCs were transfected with scleraxis knockdown or overexpression plasmids, and the results indicated that scleraxis plays a pivotal role in the differentiation of TSCs to tenocytes. In conclusion, dexamethasone inhibits the differentiation of TSCs to tenocytes by inhibiting the scleraxis gene.

1. Introduction

Local injection of glucocorticoids is among the most common methods employed to treat tendinopathies. Clinical practice and research reports have confirmed that although glucocorticoid drugs significantly relieve pain and improve motor function in the short term [1], long-term use of these drugs can trigger spontaneous rupture of the tendon [2–4]. We have also observed that in clinical practice, the long-term application of systemic glucocorticoids in patients with non-specific inflammation or autoimmune disease makes these patients more susceptible to Achilles tendon ruptures. Compared with trauma-induced tendon rupture, suture repair of glucocorticoid-induced spontaneous tendon rupture generally results in poor outcomes and the risk of future tendon rupture is rather high [5,6]. However, the reasons behind glucocorticoid-induced spontaneous tendon rupture and the low capacity for self-healing after suture repair remain unclear. No effective method exists to treat these side effects of dexamethasone.

A significant amount of basic research has been conducted assessing the effects of glucocorticoids on tendons, primarily focusing on the effect of glucocorticoids on tenocytes. For example, studies have shown that glucocorticoids reduce the mechanical properties of tendons [7]; decrease the viability [8], proliferation rate [9] and migration efficiency [10] of tenocytes; alter the fibroblast-like morphology of tenocytes and lead to the formation of disorderly arranged tenocytes [11]. Glucocorticoids also decrease the synthesis of type I collagen [9] and proteoglycans [12] in tenocytes. In addition, glucocorticoids reduce the production of metalloproteinase (MMP) 2, MMP8, MMP9, and MMP13 and enhance the synthesis of tissue inhibitor of metalloproteinase-1 (TIMP1), thereby reducing the capacity of tendons to self-repair [13].

The maintenance and repair of adult tissues rely on small populations of resident stem cells [14]. Recent studies have discovered a group of adult stem cells in human, mouse [15] and rat [16] tendons, which have been termed tendon stem cells (TSCs). TSCs display an extremely strong capacity to differentiate to tenocytes; however, TSCs may also differentiate to other types of cells of mesodermal origin, such as bone, cartilage, and fat [15,16]. Tendons are constantly in a state of dynamic equilibrium between

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microdamage and self-repair [17], and the differentiation of TSCs to tenocytes is crucial for tendon self-repair [18,19]. Therefore, if the differentiation of TSCs to tenocytes is blocked, the dynamic equilibrium will be broken, and microdamage will accumulate, increasing the risk of spontaneous tendon rupture. Zhang et al. found that the treatment of TSCs with the representative glucocorticoid drug dexamethasone resulted in decreased expression of surface markers associated with tenogenic differentiation [19]. This result suggests that dexamethasone may reduce the self-repairing capacity of the tendon and thereby increase the risk of spontaneous tendon rupture by inhibiting the differentiation of TSCs to tenocytes.

Scleraxis, which was first discovered by Alberton et al. in 1995, is a transcription factor that plays a key role in tendon development and injury repair and directs the differentiation of stem cells to tenocytes [20]. In this study, we hypothesized that scleraxis mediates the inhibition of differentiation of TSCs to tenocytes by dexamethasone. To test this hypothesis, we examined the morphological changes in TSCs following dexamethasone treatment, analyzed the expression of the tenogenic differentiation markers collagen type I and tenomodulin (TNMD) using quantitative polymerase chain reaction (qPCR) analysis, and investigated the effect of dexamethasone on the differentiation of TSCs to tenocytes by immunofluorescence and immunohistochemical analysis of the expression of an important tenocyte functional protein (type I collagen) in TSCs and human tendon tissues. We also examined the effect of dexamethasone on the expression of scleraxis mRNA and protein in TSCs at different time points using qPCR and western blot analysis, respectively. In addition, we predicted potential dexamethasone binding sites within the promoter of the scleraxis gene using TIFF searches and GeneMapper and then performed chromatin immunoprecipitation (ChIP)-PCR assays to examine binding of the glucocorticoid receptor (GR) to the scleraxis promoter and to explore the effect of dexamethasone treatment on scleraxis expression in TSCs. Lastly, we constructed a scleraxis overexpression plasmid and transfected this plasmid into TSCs to investigate the role of scleraxis in the inhibitory effect of dexamethasone on the differentiation of TSCs to tenocytes.

2. Materials and methods

2.1. Isolation and culture of rat TSCs

All experiments were approved by the Animal Research Ethics Committee of Third Military Medical University, China. Rat TSCs were isolated from Sprague–Dawley rats and cultured as described previously. Briefly, the entire intact flexor tendon was excised from both limbs of each rat following euthanasia. Only the midsubstance tissue was collected, and the peritendinous connective tissue was removed carefully. The tissues were minced in sterile phosphate-buffered saline (PBS), digested for 2.5 h at 37 °C with type I collagenase (3 mg/ml Sigma–Aldrich, St. Louis, MO, USA) and then passed through a 70-mm cell strainer (Becton Dickinson, Franklin Lakes, NJ, USA) to yield a single-cell suspension. The released cells were washed in PBS, centrifuged at 300 × *g* for 5 min and resuspended in Dulbecco's modified Eagle's medium (Gibco, Carlsbad, CA, USA) containing 10% fetal bovine serum, 100 U/ml penicillin, 100 mg/ml streptomycin and 2 mM L-glutamine (all from Invitrogen, Carlsbad, CA, USA). The isolated cells were diluted to different cell densities and cultured at 37 °C in 5% CO_2 to form colonies. At day 2 after initial plating, the cells were washed twice with PBS to remove nonadherent cells. At day 7, the cells were trypsinized and mixed together as passage 0 (P0) cells [21]. Cells from passage 1 (P1) were used for subsequent experiments. TSCs were seeded onto 6-well plates, 10-cm-diameter Petri dishes and coverslips in 24-well plates for mRNA extraction, protein extraction and ChIP-PCR and immunofluorescence, respectively. Culture medium with or without 1 μM dexamethasone (Sigma–Aldrich, dissolved in dimethyl sulfoxide (DMSO)) and mifepristone (Sigma–Aldrich, dissolved in absolute ethanol) was changed every 3 days throughout the experiments.

2.2. Overexpression and knockdown

TSCs were seeded in 6-well plates or 10-cm-diameter Petri dishes. Cell transfection was performed when the cells reached 80% confluence. The scleraxis overexpression and knockdown plasmids were purchased from GenePharma (GenePharma, Shanghai, China). Transfection was performed using the Lipofectamine 2000 reagent (Invitrogen, CA, USA) in Opti-MEM (Invitrogen, CA, USA) according to the manufacturer's protocol. TSCs were transfected for 4 h, and the transfection mixture was replaced with culture medium with or without dexamethasone (Sigma–Aldrich).

2.3. Flow cytometry

The cells were harvested and washed, and the cell suspension was adjusted to a concentration of 10^6 cells/ml in ice-cold PBS. Labeled anti-CD90-phycoerythrin and anti-CD44-FITC (Abcam) primary antibodies were added at a concentration of 6 μg/ml and incubated for 30 min at room temperature or 4 °C. The cells were washed 3 times by centrifugation at 400 × *g* for 5 min and resuspended in 500 μl–1 ml of ice-cold PBS. Analysis of fluorescence intensity was performed by flow cytometry using a Coulter Epics XL (Beckman) instrument.

2.4. Cell proliferation assay

Cell proliferation was determined using WST-8 dye (Beyotime Inst., Biotech, China) according to manufacturer's instructions. A total of 2×10^3 cells/well were seeded in four 96-well flat-bottomed plates and treated with or without 1 μM dexamethasone for 1 day, 3 days, 5 days, or 7 days. Following the addition of 10 μl of WST-8 dye to each well, the cells were incubated at 37 °C for 1.5 h, and the absorbance was determined at 450 nm using a microplate reader.

2.5. qPCR

The mRNA expression levels of scleraxis, type I collagen and TNMD were determined using qPCR. Total RNA was extracted from the cells using TRIzol reagent according to the protocol provided by the manufacturer (TaKaRa, Japan). cDNA was synthesized from total RNA using a Superscript III first-strand synthesis kit (TaKaRa, Japan). qPCR was performed using a SYBR Green RT-PCR kit (TaKaRa, Japan) and an ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, Foster City, CA, USA). Expression levels were calculated relative to the expression of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer sequences used in these experiments are listed in Table 1.

2.6. Protein extraction and western blotting

Cells were scraped and homogenized in lysis buffer (50 mM Tris–HCl, pH 8.0, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 150 mM NaCl) containing a mixture of proteinase inhibitors (Thermo Fisher Scientific Inc., Rockford, IL, USA). Protein concentrations were measured using a BCA protein assay kit (Thermo Fisher Scientific Inc.). Protein samples (30 μg/lane) were resolved by