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TSG-6 mediates the effect of tendon derived stem cells for rotator cuff healing

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Abstract. – BACKGROUND: Bone marrow stem cells (MSCs) were able to reduce fibrovascular tissues formation via TNF alpha-stimulated gene/protein 6 (6TSG-6) in various animal models. At the same time, tendon-derived stem cells (TDSCs) were able to promote rotator cuff healing; however, the mechanism is still unknown.

AIM: To investigate the role of TSG-6 in the treatment of rotator cuff healing with TDSCs.

MATERIALS AND METHODS: 45 rats underwent unilateral detachment and repair of the supraspinatus tendon. 15 animals received TDSCs in a fibrin glue carrier(Group A), 15 received TSG-6 silenced TDSCs (Group B), and 15 received fibrin glue for control (Group C). Animals were sacrificed at 4 weeks and evaluated for the biomechanical testing. Statistical analysis was performed with an independent t test with significance set at $p = 0.05$.

RESULTS: The ultimate stress was greater in the TDSCs group (4.91±1.41 N/mm^2) as compared with the Control group (2.99±1.04 N/mm^2) ($p < 0.05$). However, when silent the expression of TSG-6, the TSG-6 silenced group (3.36±0.96 N/mm^2) showed no benefit over the control group ($p = 0.32$).

CONCLUSIONS: TSG-6 mediates the function of TDSCs to improve the structure and the attachment strength of the healing tendon-bone interface.

Key Words:
TSG-6, TDSCs, Tendon derived stem cells, Tendon-bone interface.

Introduction

Rotator cuff tears are commonly happened in athletic settings and the elderly population, with marked pain and functional impairment. Although rotator cuff repair technique improved significantly over the past years, there is still concern over the tendon's ability to heal to bone in the postoperative period[1-3]. The tendon-to-bone insertion site rarely regenerated. Instead, the reconstructed structure forms a mechanically inferior fibrovascular tissue[4,5].

Tendon-derived stem cells (TDSCs) have recently been identified within tendon tissues[6], which are able to promote the regeneration of the tendon-to-bone insertion site[7,8]. Nevertheless, how TDSCs promote recovery is unknown. The proliferation and differentiation of TDSCs into tenocytes may play an important role. But more importantly, the transplanted cells contribute to the tendon healing by producing tropic paracrine factors, as the number of TDSCs decrease with time[7,9].

Recent reports based on bone marrow stem cells (bMSCs) have demonstrated that many, but not all, of the therapeutic effects of the cells are explained by MSCs being activated by signals from injured tissues to secrete anti-inflammatory factors, as most of the intravenous infused cells were found trapped in the lungs as microemboli[10,11]. In various animal models (lung injury, corneal injury, peritonitis, myocardial infarction), the TNF-α stimulated gene/protein 6 (TSG-6) was found to play the role of anti-inflammatory[10,12-15].

Given above, we hypothesized the TDSCs may promote the tendon-to-bone healing by secreting TSG-6 to reduce the inflammatory and the fibrovascular tissue, which results to a more resembled the native insertion site in terms of composition (regeneration of the fibrocartilaginous transition zone), structure (more organized collagen fibers) and stronger tendon-to-bone fixation (evidenced by biomechanical testing).

Materials and Methods

Study design

A total of 55 Lewis rats (Shanghai Super, B&K Laboratory Animal Corp. Ltd.) were used in this study. Ten rats were used for TDSCs harvest, and 45 rats underwent unilateral detachment and re-

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pair of the supraspinatus tendon. This study was approved by the Ethics Committee of Shanghai Tenth People's Hospital. Lewis rats were chosen because they are considered syngeneic as they are inbred, which limits the risk of graft rejection. Animals were randomized into 3 groups with 15 rats per group: TDSCs in a fibrin glue carrier (10^6 cells), TSG-6 silenced TDSCs in a fibrin glue carrier (10^6 cells) and the blank control group. The rats were sacrificed at 4 weeks for biomechanical testing to determine the structural and material properties of the repaired tendon.

Tendon Derived Stem Cells Harvest and Culture

Firstly, obtain the mid-substance of the tendon, remove the peritendinous connective tissue, cut the tendon into pieces, and digested with type I collagenase and dispase. Then, the digestates are centrifuged and resuspended in low-glucose Dulbecco's modified Eagle medium, supplemented with 10% fetal bovine serum, 10 U/mL of penicillin, 100 mg/ml of streptomycin, and 2 mML-glutamine (complete basal culture medium). After diluting the suspension to 10 cells/ml, the cells are plated at low density, and cultured at 37°C in 5% carbon dioxide to form colonies. We also use 70-μm pore-size nylon filters to recover the isolated cells. On day 2 after initial plating, the cells are washed twice with phosphate-buffered saline (PBS) to remove the non-adherent cells. A portion of tendon-derived cells attach to the plate and remain quiescent for 3-5 days before undergoing rapid division to form colonies. On days 7-10, the cells are trypsinized and mixed together as passage 0.

Transfections with TSG-6 siRNA

Target TDSCs for the transfections were prepared with viable passage 1 TDSCs that were plated at 50,000 cells/well in cell culture media (CCM) in 6-well plates. After incubation for 1 day, cells were transfected with 10 or 20 nM siRNA for TSG-6 (sc-39819; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or RNAi-negative control (Stealth RNAi negative Control; Invitrogen, Carlsbad, CA, USA) using a commercial kit (Lipofectamine RNAiMAX reagent; Invitrogen). Six hours later, the medium was replaced with 3 ml per well of CCM lacking antibiotics, and hMSCs were incubated for 16-20 hr.

Surgical Procedure

Split the deltoid and detach the supraspinatus from its footprint. A #15 blade knife was used to clear the fibrocartilage completely of the greater tuberosity leaving a bleeding bone directly. A stitch was placed into the supraspinatus tendon. A 22-gauge needle was used to create 2 bone tunnels at the anterior and posterior margins of the insertion site. The sutures in the tendon were then passed through the tunnels. Finally, fill the interspace between the bone and the tendon with fibrin glue carrier, which contains at least 10^6 TDSCs[16-18]. The control group injects just fibrin glue.

Biomechanical Testing

At weeks 4, twelve animals per group were euthanized for biomechanical testing. The supraspinatus tendon was isolated carefully. Make sure the supraspinatus muscle belly and the sutures were detected away from the tendon. Digital calipers were used to measure the cross-sectional area of the supraspinatus tendon to bone interface. The specimen was then transferred to a custom-made uniaxial testing system. Each specimen was initially preloaded to 0.10 N. The tendon was then loaded at a rate of 14 microns/s until the tendon repair failed, at which point the maximum load at failure were recorded. A 1-micron resolution micrometer was used to measure the displacement. Ultimate stress at failure was defined as dividing the ultimate force at failure by the cross-sectional area.

Statistical Analysis

The null hypothesis is that TSG-6 plays an important role in the tendon-to-bone healing induced by TDSCs. The primary outcome is the biomechanical testing of the tendon-to-bone attachment. A power analysis (PASS 11) was performed with the data of our preliminary experiment that compared the difference of the ultimate stress between the TDSCs and the control group. Using these estimations, a power of 0.80 is achieved using at least 12 specimens per group with a = 0.05 for biomechanical testing. The statistical analysis was performed using the SPSS software, version 13.0 (SPSS Inc., Chicago, IL, USA). The data between the groups were compared with independent sample *t* tests. A difference with a *p* value of less than 0.05 was interpreted as significant.

Results

TDSCs Harvest and Culture

TDSCs were cultured and found to be able to differentiated into differentiate into chondrocytes, osteocytes, and adipocytes (Figure 1).

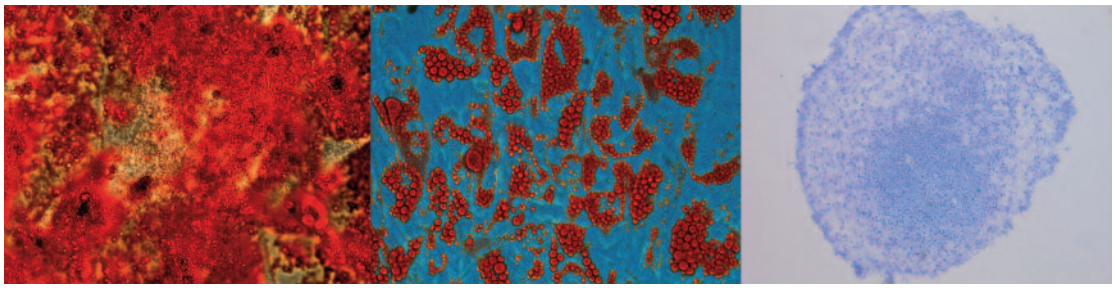

Figure 1. Multiple Differentiation potential of TDSCs.

In Vitro Confirmation of MSC Transduction

Quantitative real-time PCR confirmed that the *in vitro* TSG-6 gene expression was 115-fold greater in the transduced TDSCs when compared with the untransduced TDSCs (Figure 2).

Biomechanical Testing

At 4 weeks, the ultimate stress was greater in the TDSCs group (4.91±1.41 N/mm^2) as compared with the Control group (2.99±1.04 N/mm^2) ($p < 0.05$). However, when silent the expression of TSG-6, the TSG-6 silenced group (3.36±0.96 N/mm^2) showed no benefit over the control group ($p = 0.32$) (Figure 3).

Discussion

In the this study, we have demonstrated that the TDSCs group showed higher ultimate stressSI than the control group, while the TSG-6 silenced TDSCs group showed similar ultimate stressSI. Consequently, we were able to support our primary hypothesis that TSG-6 played an essential role for the regeneration of the tendon-to-bone insertion site induced by TDSCs.

Surgical interventions are always needed to reconstruct the rotator cuff insertion site, however, it rarely regenerated. Previous researches found MSCs was able to promote the regeneration of the tendon to bone interface[7,8]. Same results were found in this work that the TDSCs group exhibited higher ultimate stress than the control group. Controversy continues with regard to the mechanism of the function of TDSCs. The proliferation and differentiation of TDSCs into tenocytes may play an important role. The transplanted cells contribute to tendon healing by producing tropic paracrine factors that promote healing, rather than direct differentiation to tenocytes. Nowadays, consensus appears to have been reached that the key point for the tendon-to-bone healing is to re-

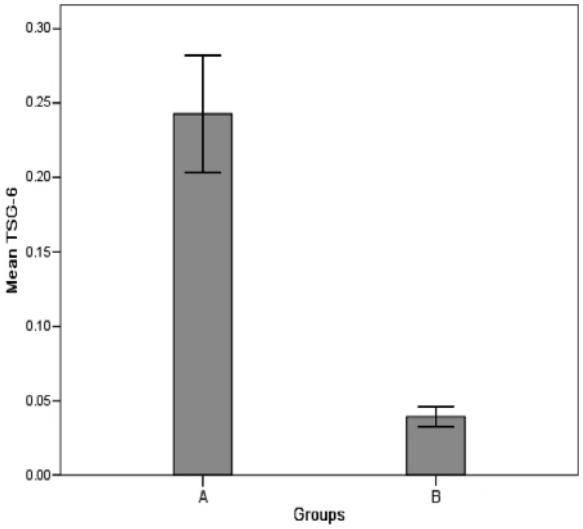

Figure 2. The comparison of the expression of TSG-6 gene between the groups.

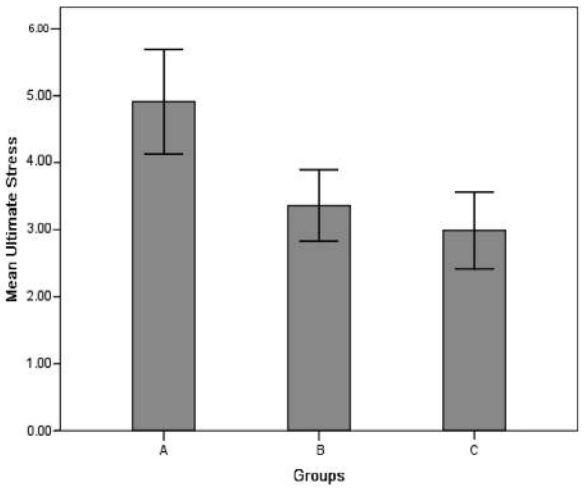

Figure 3. The comparison of the ultimate stress between the groups.

duce the formation of mechanical inferior fibrovascular tissue at the repair site.

Formidable inflammatory and immune response systems protect us against various microorganisms. However, excessive inflammatory and immune responses may be a threat to various diseases like myocardial infarction (MI) and stroke[19], in which fibrovascular tissue forms and damages the function. Recent reports indicate that bMSCs serves as important guardian cells for modulating inflammation[10,12-15,20]. In a series of experiments performed with a model of bleomycin-induced lung injury, intravenous (i.v.) infusion of bMSCs decreased the inflammatory response to bleomycin and prevented the lungs from developing fibrosis[21,22]. Another study showed i.v. injection of bMSCs significantly reduced the inflammatory response to permanent ligation of the anterior descending coronary artery and subsequently reduced the size of the myocardial infarcts[10,11]. Though bMSCs that i.v. infused are mostly trapped in the lung, there's still beneficial effects in repairing tissues in distal organs. A negative feedback loop in which damage-associated molecular patterns (DAMPs) from injured tissues and macrophages activate MSCs to secrete the multifunctional anti-inflammatory protein TNF-α stimulated gene/protein 6 (TSG-6). The TSG-6 then reduces nuclear factor-κB (NF-κB) signaling in the resident macrophages and thereby modulates the cascade of proinflammatory cytokines. The bMSCs with a siRNA knockdown of the TSG-6 gene had little or no effect in the MI model. Also, the i.v. administration of recombinant human TSG-6 (rhTSG-6) had about the same beneficial effect as bMSCs.

Conclusions

TSG-6, a therapeutic protein produced by TDSCs in response to injury signals, prevented fibrovascular tissue formation in the tendon-to-bone interface by suppressing inflammation.

Conflict of Interest

The Authors declare that there are no conflicts of interest.

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