Cytotherapy, 2015; 17: 215–223

International Society for Cellular Therapy
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In vitro mutual interaction between tenocytes and adipose-derived mesenchymal stromal cells

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Abstract

Background aims. Tendon is a complex tissue with a reduced regenerative ability. Nowadays, little or nothing is known about the regenerative effect of adipose-derived mesenchymal stromal cells (ADSCs) on tendons. *Methods.* The study aimed to evaluate the *in vitro* mutual interaction of ADSCs and tenocytes in standard culture conditions and a microwound healing model. Tenocyte viability, microwound recovery and the expression of genes encoding for the main extracellular matrix components and ADSC viability, differentiation and growth factor gene expression were evaluated. *Results.* The effects of ADSCs on tenocytes were observed more in the microwound healing model, in which the rate of microwound healing and the expression of decorin, tenascin and collagens were significantly increased. The influence of tenocytes on ADSCs was also found in standard culture conditions: ADSCs were directed toward a tenogenic lineage, and growth factor expression increased. *Conclusions.* This study clarifies some aspects of the mutual interaction of ADSCs and tenocytes and provides *in vitro* evidence for a possible future application of ADSCs as a therapeutic strategy for tendon repair.

Key Words: *adipose-derived mesenchymal stromal cells, gene expression, mutual interaction, tendon healing, tenocytes*

Introduction

Tendon is a complex tissue that connects and translates motion from muscle to bone. It is composed of a dense extracellular matrix (ECM) of collagen fibrils, produced by specialized tendon-specific fibroblasts (tenocytes) [1].

Tendon inflammation, degeneration and then rupture are due to several factors, including overuse and sudden increased strain. The avascular nature, relatively poor content of tenocytes (highly differentiated cells with limited proliferation rate) and reduced cellular metabolic rate are responsible for the poor tendon self-healing ability that results in poor tendon quality and reduced mechanical properties [2]. The repair process is slow and a scar tissue forms with reduced mechanical properties, thus making it prone to re-injury [3]. If not treated, knee tendon damage might cause instability and lead to the early onset of osteoarthritis, pain and disability [4]. Conventional therapies aim to restore the biological and biomechanical status of the native tissue. These therapies include drugs, physiotherapy and surgery, which mainly involves autografts, allografts, xenografts or the use of prosthetic devices (in severe tendon injuries) [5]. Combined donor site morbidity, high failure rates and the risk of re-injury impede long-term functional recovery. Thus, finding a successful therapeutic approach is still a challenge and a biological strategy is required to improve the quality of the tissue. Mesenchymal stromal cells (MSCs) have already been used in the regeneration of several musculoskeletal tissues [6], thanks to their ability to differentiate *in situ* toward the desired cell lineages. Moreover, MSCs are able to secrete trophic molecules, such as growth factors (GFs) and chemotactic molecules, which can recruit additional reparative cells into the lesion site [7]. Bone marrow–derived MSCs (BMSCs) are the most commonly used cells in tissue engineering, and the implantation of BMSCs into tendon injury has provided promising results in several *in vivo* studies, thus indicating that the microenvironment and cellular interactions play an

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(Received 1 August 2014; accepted 13 October 2014)

ISSN 1465-3249
http://dx.doi.org/10.1016/j.jcyt.2014.10.006

important role in MSC differentiation [8]. Among other potential sources of MSCs [9], adipose-derived MSCs (ADSCs) appear to be the most promising stem cell population to have clinical relevance as an alternative for tendon repair. In addition, it has been reported that ADSCs have numerous advantages over BMSCs because they are readily available, easy to extract and isolate and do not produce postoperative complications [10]. The *in vitro* growth and efficacy of ADSCs has been reported in preclinical and clinical studies [11]. Some previous *in vitro* studies showed the ability of ADSCs to undergo tenogenic differentiation in the presence of appropriate biological stimuli, tensile strain, oxygen tensions [12–15], scaffolds [16,17] and when co-cultured with tenocytes as in a 3-dimensional high-density system [18,19].

Several studies have analyzed the molecular events that occur after tendon injury and during the healing process [20–23], but preclinical research on the ability of ADSCs to regenerate tendons is still in its infancy. Until now, the few *in vivo* studies that have shown the regeneration potential of ADSCs on tendon transections and tendonitis have not clarified the exact molecular mechanisms of action [24–28].

Starting from the hypothesis that there might be a mutual interaction between ADSCs and tenocytes, the main purpose of the present study was to verify whether ADSCs improve tenocyte viability and gene expression in an *in vitro* indirect co-culture system that allows the exchange of soluble factors between the two cell types without cell-cell contact. An *in vitro* tenocyte microwound healing model was also adopted as described in a previous study [29]. In addition, tenocyte influence on ADSCs was assessed in both standard culture conditions and in the microwound healing model.

It was observed that ADSCs not only improved the *in vitro* tenocyte viability and gene expression and the microwound repair, but also produced a mutual effect between tenocytes and ADSCs in both culture conditions.

Methods

The study protocol fell within the scope of other projects on bone, not affecting tendons, adipose tissue and ADSCs. These studies were approved by the Rizzoli Orthopaedic Institute Ethical Committee and by the Italian Ministry of Health and were performed strictly following the current laws on animal experiments.

Cell isolation

The Achilles tendons of three healthy 5-month-old female Sprague Dawley rats were dissected under sterile conditions. The explants were cut into 2 × 2-mm fragments and were cultured in growth tenocyte medium (TM), composed of a 1:1 mixture of HAM'S F12:Dulbecco's modified Eagle's medium (Sigma Aldrich, St Louis, MO, USA), 10% fetal bovine serum (Lonza, Verviers, Belgium), 1% penicillin-streptomycin solution (Gibco, Invitrogen, Carlsbad, CA, USA) and 25 μg/mL ascorbic acid (Sigma Aldrich). The cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere. After 7 days, the tenocytes were observed in the cultures and then were grown until 90% confluence. The tenocytes were used for the experimental study after the first passage (P1).

Abdominal adipose tissue (7.73 ± 2.53 mg), harvested from the same donors, was digested with 0.075% collagenase II (Sigma Aldrich). The enzymatic reaction was stopped by the addition of complete medium (CM), that is, Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Lonza), 100 U/mL penicillin, 100 μg/mL streptomycin (Gibco, Invitrogen) and 5 μg/mL plasmocin (InvivoGEN, San Diego, CA, USA). The pellet was treated with 160 mmol/L NH_4Cl (Sigma Aldrich) for erythrocyte lysis. The nucleated cells were then seeded in CM. At subconfluence, the adherent cells (ADSCs) were used for the experiments. Before the setup of the indirect co-culture system, the ADSCs were characterized through the evaluations of surface epitopes, clonogenicity and osteogenic differentiation [21].

Indirect co-culture (standard culture condition)

The tenocytes were plated at the bottom of 24-well plates at a concentration of 1×10^4 cells/well in TM. ADSCs were seeded in 24-well 0.4-μm pore size transwells (Millipore, Carrigtwohill, Country Cork, Ireland) at a concentration of 3×10^3 cells/transwell in CM. At nearly 80% of confluence, ADSC-containing transwells were transferred into the wells with tenocytes and the co-cultures were maintained for up to 7 days in CM. Single cultures of tenocytes or ADSCs were used as controls. All the experiments were performed in triplicate and evaluations were performed after 24 hours and 3 days and 7 days of cultures.

In vitro tendon microwound healing model

In parallel, an *in vitro* microwound healing model was performed [29]. Briefly, once a confluent monolayer of tenocytes had been grown, a sterile Eppendorf tip was used to produce an artificial wound of 900-μm width by scraping the cell layer (time zero, T0). The transwells containing ADSCs were then transferred to the wells and evaluations were performed after 1, 4, 8, 24 and 48 hours (T1, T4, T8, T24 and T48). Empty transwells were transferred to the wells as controls, and all the experiments were performed in triplicate.