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Use of biomimetic microtissue spheroids and specific growth factor supplementation to improve tenocyte differentiation and adaptation to a collagen-based scaffold *in vitro*

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ABSTRACT

Tenocytes represent a valuable source of cells for the purposes of tendon tissue engineering and regenerative medicine and as such, should possess a high degree of tenogenic differentiation prior to their use *in vivo* in order to achieve maximal efficacy. In the current report, we identify an efficient means by which to maintain differentiated tenocytes *in vitro* by employing the hanging drop technique in combination with defined growth media supplements. Equine tenocytes retained a more differentiated state when cultured as scaffold-free microtissue spheroids in low serum-containing medium supplemented with L-ascorbic acid 2-phosphate, insulin and transforming growth factor (TGF)-β1. This was made evident by significant increases in the expression levels of pro-tenogenic markers collagen type I (*COL1A2*), collagen type III (*COL3A1*), scleraxis (*SCX*) and tenomodulin (*TNMD*), as well as by enhanced levels of collagen type I and tenomodulin protein. Furthermore, tenocytes cultured under these conditions demonstrated a typical spindle-like morphology and when embedded in collagen gels, became highly aligned with respect to the orientation of the collagen structure following their migration out from the microtissue spheroids. Our findings therefore provide evidence to support the use of a biomimetic microtissue approach to culturing tenocytes and that in combination with the defined growth media described, can improve their differentiation status and functional repopulation of collagen matrix.

1. Introduction

Tendon lesions in equine and human athletes are mainly the result of overstrain injury rather than percutaneous trauma, where the athletic discipline generally predisposes specific tendons to injuries. In the racehorse and event horses, injuries occur most frequently in the suspensory ligament and the superficial digital flexor tendon (SDFT) [1]. In humans, tendinopathy is most commonly diagnosed in the Achilles, patellar, rotator cuff and medial/lateral elbow tendons and accounts for 30–50% of all sports-related injuries [2]. As such, there is an ever growing need for more effective therapies with which to combat tendon injuries and the ensuing degeneration associated with such trauma. The use of cell-based tissue engineering approaches are fast emerging as alternative therapeutic strategies for the management of tendon injury both in humans and animals [3]. Indeed, a growing number of reports now exist in which multipotent stromal cells (MSCs) isolated from various sources have been successfully implemented in the treatment of both experimentally induced tendon defects [4–7], as well as in clinical cases of tendinopathy [8,9]. The use of MSCs primarily relies on their ability to differentiate into fully functional tendon cells, termed tenocytes. As such, investigators have also sought to utilize mature tenocytes directly as an

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additional means by which to regenerate tendon tissue as evidenced by the initiation of human clinical trials [source: ClinicalTrials.gov, Trial Number: NCT01343836 and source: ClinicalTrialsRegister.eu, Trial Number: 2010-021869-73] with the aim to evaluate the efficacy of autologous tenocytes in treating chronic tendinopathy.

Tenocytes are fibroblast-like cells derived from embryonic mesenchyme and form a three dimensional network of cell processes throughout the extracellular matrix linked by gap junctions [10]. These cell processes not only connect tenocytes with each other, but also enclose the collagen bundles. It is thought that this close relationship between tenocytes and collagen fibril bundles enables cellular load sensing and coordination of response to load. Characterization of tenocyte gene expression has revealed several markers considered to be essential for their development into fully functional tendon-forming cells, of which tenomodulin (TNMD) may be considered one of the most important and well studied [11–13]. TNMD is a type II transmembrane protein that is mainly expressed in dense connective tissues, and is generally regarded as being a late marker of tendon development [14]. *TNMD* gene expression is positively regulated during tendon development by scleraxis (SCX) [14,15], a transcription factor considered essential for efficient tendon differentiation [16]. However, both TNMD and SCX have also been detected in tissues from sources other than tendon, thereby bringing into question their reliability as specific markers of tendon [17]. Despite this fact, both TNMD and SCX still serve as a means by which to gauge the differentiation status of tenocytes isolated from tendon tissue.

It has previously been reported that *in vitro*, mature tenocytes have a reduced tendency to express *TNMD*, as well as collagen type 1 (*COL1*), and lose their elongated morphology when cultured for extended periods [18–20]. This so called dedifferentiation of cultured tenocytes is not only considered to be potentially detrimental to their efficiency as a cell-based therapy in tendon repair, but also to their usefulness as an *in vitro* cell system for developing and testing alternative treatments. In this regard, various studies have been undertaken with an aim to improving the genotype and phenotype of isolated tenocytes. Of critical importance in this regard, is the growth medium and its components used to maintain tenocytes in culture. Several investigators have identified transforming growth factor (TGF)-β and insulin-like growth factor (IGF)-1 as being potent inducers of tenogenic differentiation in both MSCs and tenocytes [21–26], allowing cells to maintain a tenogenic genotype and phenotype *in vitro*. Furthermore, the transfer of cultured tenocytes to high density, three-dimensional (3D) growth environments also appears to stimulate differentiation and prevent cellular dedifferentiation to some degree [21,25–27].

The primary focus of the present study was to determine whether equine tenocytes could generate self-assembled gravity-enforced 3D microtissue spheroids *in vitro*, and if so, could this improve their tenogenic differentiation status over that of tenocytes cultured under standard 2D conditions. Furthermore, various combinations of growth factor supplementation were evaluated in terms of their ability to influence the genotype and/or phenotype of equine tenocytes maintained as 2D or 3D cultures. Equine tenocytes cultured as hanging drops formed microtissue spheroids, and after 2 and 6 days of culture, displayed significantly greater levels of several recognized tendon cell markers as compared to monolayer cultures as determined by RT-qPCR. Moreover, the use of low serum growth media supplemented with TGF-β1 in combination with insulin and ascorbic acid enhanced these effects, and when used to stimulate microtissue-derived tenocytes embedded in collagen scaffolds, induced phenotypic features typical of differentiated tenocytes. Our findings therefore suggest that scaffold-free biomimetic microtissue spheroids, in combination with specific chemical compositions of growth media, may represent an efficient means by which to maintain equine tenocyte differentiation *in vitro*. Such a system may therefore allow for tendon cell and tissue biology to be studied *in vitro* using conditions that more closely simulate the *in vivo* situation and could potentially offer an alternative strategy for stimulating tendon tissue formation *in vivo*.

2. Materials and methods

2.1. Animals

Tenocytes were isolated from the superficial digital flexor tendon of Warm blood horses, aged between 2 and 4 years, which were naturally destroyed for clinical reasons other than orthopaedic disease and where owner consent was obtained.

2.2. Isolation and culture of equine tenocytes

Tenocytes were isolated in accordance with previously established methodologies [17,20]. The mid-metacarpal region of the superficial digital flexor tendon was harvested under sterile conditions. All tendons were free of pathology on clinical and post mortem examination. After removal of the peritendineum, tendon sections of approximately 1 cm in length were cut into 2 mm^3 pieces and subjected to 0.2% collagenase NB4 (Roche Diagnostics, Rotkreuz, Switzerland) and 0.3% dispase II (Roche Diagnostics) digestion overnight in phosphate-buffered saline (Life Technologies, Zug, Switzerland) containing 5% penicillin/streptomycin (Life Technologies) on an orbital shaker at 37 °C. Digested tissue was filtered through a 70 μm cell strainer, centrifuged and cells re-suspended in normal growth medium (GM) consisting of Dulbecco's modified eagle medium (DMEM-high glucose) supplemented with 10% fetal bovine serum (FBS) and penicillin/streptomycin (all from Life Technologies). Supernatant was replaced after 1 day and thereafter every 3–4 days with fresh GM and cells were used between passage 4 and 7 unless otherwise stated. The potential contamination of tenocyte cultures with MSCs was assessed through the use of osteogenic and adipogenic induction assays according to previously described methodologies [28]. Briefly, monolayer cultures of equine tenocytes were incubated for 14 days with either osteogenic or adipogenic induction medium and stained with Alizarin red or Oil Red O in order to assess osteogenic and adipogenic differentiation respectively. Additionally, MSCs isolated from equine adipose tissue were also incubated with either osteogenic or adipogenic induction medium and served as a positive multipotent cell control.

2.3. Stimulation of equine tenocytes

For 2D monolayer cultures, equine tenocytes seeded at 6×10^4 cells/cm^2 and stimulated with GM, or DMEM-high glucose supplemented with either; (i) 1% FBS and TGFβ1 (10 ng/ml; Peprotech, London, UK); (ii) 1% FBS, TGFβ-1 (10 ng/ml) and IGF-1 (50 ng/ml; Peprotech, London, UK) (termed tenogenic differentiation medium-I; TDM-I) or with; (iii) 1% FBS, 50 μM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, 0.5 μg/ml insulin (all from Sigma–Aldrich, Buchs, Switzerland) and 10 ng/ml human TGF-β1 (termed tenogenic differentiation medium-II; TDM-II). In order to generate tenocyte (TC)-microtissue spheroids, cells were seeded as hanging drops in Terasaki plates (VWR International, Dietikon, Switzerland) in GM, TDM-I or TDM-II according to previously published methodologies [29]. Briefly, equine tenocytes were adjusted to 4×10^4 to 2×10^5 cells/ml in culture medium and a 25 μl cell suspension transferred to individual wells of a Terasaki plate. The plate was then sealed with a lid and inverted in order to