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Effects of substrate stiffness on the tenoinduction of human mesenchymal stem cells

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ABSTRACT

Extracellular matrix modulus plays an important role in regulating cell morphology, proliferation and differentiation during regular and diseased states. Although the effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis, there has been relatively little investigation on the effects of this phenomenon on tenogenesis. Furthermore, relative roles of topographical factors (matrix alignment vs. matrix modulus) in inducing tenogenic differentiation is not well understood. In this study we investigated the effects of modulus and topographical alignment of type I collagen substrate on tendon differentiation. Type I collagen sheet substrates with random topographical alignment were fabricated with their moduli tuned in the range of 0.1, 1, 10 and 100 MPa by using electrocompaction and controlled crosslinking. In one of the groups, topographical alignment was introduced at 10 MPa stiffness, by controlled unidirectional stretching of the sheet. RT-PCR, immunohistochemistry and immunofluorescence results showed that mimicking the tendon topography, i.e. increasing the substrate modulus as well as alignment increased the tenogenic differentiation. Higher substrate modulus increased the expression of COLI, COLIII, COMP and TSP-4 about 2–3-fold and increased the production of COLI, COLIII and TSP-4 about 2–4-fold. Substrate alignment up regulated COLIII and COMP expression by 2-fold. Therefore, the tenoinductive collagen material model developed in this study can be used in the research and development of tissue engineering tendon repair constructs in future.

Statement of Significance

Although the effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis, there has been relatively little investigation on the effects of this phenomenon on tenogenesis. Furthermore, a relative role of topographical factors (matrix alignment vs. matrix modulus) in inducing tenogenic differentiation is not well understood. We investigated the effects of modulus and topographical alignment of type I collagen substrate on tendon differentiation. This study showed mimicking the tendon topography, i.e. increasing the substrate modulus as well as alignment increased the tenogenic differentiation. Therefore, the tenoinductive collagen material model developed in this study can be used in the research and development of tissue engineering tendon repair constructs in future.

1. Introduction

Modulus of the extracellular matrix plays an important role in regulating cell morphology, proliferation, differentiation during regular and diseased states. For example, 1 kPa stiffness favors differentiation of MSCs into neuronal-like cells, 10 kPa elasticity promotes myogenic differentiation, and a 100 kPa matrix stiffness stimulates osteogenic differentiation [1]. The effects of substrate topography and modulus on MSC differentiation are well known with respect to osteogenesis and adipogenesis [1–6]. However, there has been relatively little investigation of this phenomenon with respect to tenogenesis. Sharma et al. studied the effect of tenogenesis on collagen coated polyacrylamide substrate with

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modulus gradient in a modulus range less than 0.1 MPa [5,7]. On the other hand, it is known that the native modulus of the tendon tissue to be as high as 1200 MPa [8,9]. Therefore, studies to date do not cover a modulus range that is more compatible with the tendon tissue environment.

The literature also reports that unidirectionally aligned fibrous topography as another tenogenic differentiation cue. Tong et al. reported that tendon cell differentiation of hMSCs occurs on collagen coated PDMS substrates which replicate native tendon surface [10]. Yin found that aligned substrate promotes tenogenesis in tendon/stem progenitor cells (TPSC) but did not evaluate MSCs [11]. Furthermore, aligned electrochemically compacted collagen fiber, electrospun polyurethane (PU), silk fibroin, poly (l-lactic acid) and two-dimensional (2D) microgrooved surface showed elongated cell morphology and tendon related ECM formation [12–19].

To date, the effects of matrix alignment and matrix modulus on tenogenesis have been investigated separately and the synergy between the two variables in terms of inducing tenogenic differentiation is not studied. Our research group created a collagen based material platform to investigate the effect of modulus in a broad range of 0.1 MPa to 100 MPa while controlling the topographical alignment random or unidirectional. In this method, collagen solutions are transformed to highly dense randomly oriented collagen sheets by electrochemical compaction induced by planar electrodes [20–22]. Matrix alignment is introduced in these sheets by controlled unidirectional stretch of the sheets as molecular alignment lacks when planar electrodes are used to fabricate sheets [21]. The aim of the current study is to study the effects of matrix modulus and alignment on tenogenic differentiation of human MSCs. Importantly, a broad modulus range of 0.1–100 MPa is covered logarithmically for the first time in the literature. Elucidation of the mechanism of topographically induced tenogenesis in human MSCs as such will assist in optimizing materials to produce scaffolds for tendon repair and may provide insight into the differentiation mechanisms of MSCs and other stem cells in vivo.

2. Materials and methods

2.1. Collagen sheet fabrication by electrochemical compaction

Electrocompacted collagen sheet was fabricated by the method as described before [21]. Briefly, twofold diluted (with RNAase/DNAase free water) acid soluble monomeric Type-I collagen solution (Collagen Solutions, San Jose, CA; 6 mg/ml) was adjusted for pH 8–10 using 1 N NaOH and dialized using ultrapure water for 18 h. The collagen solution was then used for electrocompaction.

Collagen sheet was fabricated in sheet form by electrocompaction method described before [20,23]. Briefly, a $30 \times 10 \times 1.5$ mm rectangular window was cut in a plastic piece. The plastic piece was then sandwiched between two planar carbon electrodes by filling the rectangular window with the collagen solution. A 30 VDC electrical potential was applied across the electrodes for 2 min. Collagen molecules are electrophoretically mobilized and compacted due to pH gradients established between the two electrodes under the mechanisms detailed in an earlier publication [24]. 100–200 μm thick collagen sheets of 30×10 mm dimensions were generated by the electrocompaction. Collagen molecules are randomly oriented within the plane of the sheet. In one of the experimental groups, collagen molecules were unidirectionally aligned by stretching the sheet using a motorized mechanical device as described earlier [21] (Figs. 1 & 2). Collagen sheet samples were incubated in phosphate buffered saline (PBS) for six hours at 37 °C to induce fibril formation and treated with 2-propanol solution for 12 h. Different levels of modulus were attained by crosslinking the sheets as will be described.

2.2. Synthesis of collagen gel

Elastically most compliant group was the standard collagen gel which was not subjected to electrocompaction. Acid soluble monomeric collagen solution was mixed with 10× PBS at 8:1 ratio. pH was adjusted to 7.4 using 0.1 N NaOH. The collagen solution was then placed in at 37 °C for 1 h by pouring in a petri dish to form gel. The gel was then crosslinked with genipin in 95% ethanol for 3-days as detailed in Table 1.

2.3. Experimental groups

Four levels of modulus were targeted (0.1, 1, 10 and 100 MPa) for randomly aligned collagen sheets to investigate the effect of modulus on tenoinduction. These modulus levels were attained by changing the crosslinking protocols [Table 1]. Crosslinking was carried out by genipin (Wako Chemical, Japan) in 90% v/v ethanol solution at 37 °C. The fifth group was a unidirectionally aligned collagen sheet group at 10 MPa along the axis of alignment to assess the effects of anisotropy. Samples were treated with per acetic acid (Sigma Aldrich, USA) ethanol solution (2% Acetic acid + 96% Ethanol) after the cross linking to bleach out extra genipin which may keep crosslinking the samples.

2.4. Imaging of 10 MPa compacted collagen sheets for molecular alignment

Polarized optical microscope (Olympus BX51, Melville, NY, USA) was used to assess the alignment of collagen molecules at 10 MPa aligned group as described before [21]. Briefly, as collagen is a positive birefringent material, the aligned molecules shows blue interference color along the slower axis of first order wavelength gypsum plate [25]. Therefore, molecular alignment is indicated by blue in the Compensated Polarized Imaging (CPI) and lack of alignment is indicated by Magenta color. Alignment was also confirmed by scanning electron microscopy (SEM-FEI Nova Nanolab 200, Hillsboro, Oregon).

2.5. Assessment of mechanical properties

Collagen sheet samples described in Table 1 were tested at a strain rate of 10 mm/min under monotonic tension (Rheometrics Inc., NJ) (n = 6–8 samples per group) to assess the attainment of targeted modulus values. Samples were cut into 20×2 mm strips and hydrated in deionized water for 30 min before testing. A custom-made micrometer was used to measure the thickness of the sheet samples where the micrometer closes an electrical circuit upon contact with the surface of the sample. Stress–strain curves were constructed using the load–displacement data and sample geometry. Modulus was calculated from the slopes of the linear regions of stress strain curves.

2.6. Effect of matrix modulus and alignment on tenogenic differentiation of human MSCs

Samples were sterilized in 70% ethanol for 4 h, washed in 1× PBS and placed into ultralow attachment 24 well plates (Corning) (n = 3 wells/group). Human mesenchymal stem cells (MSCs) (Lonza, Walkersville, MD) at passage 5 were seeded at a density of 20,000 cells/cm^2. The culture medium composed of alpha MEM (Invitrogen) supplemented with 10% MSC-Qualified FBS (Invitrogen), 1% penicillin/streptomycin and 50 μg/mL ascorbic acid. Cells were cultured for 21 days with medium changes every 3 days. Tenogenic differentiation was assessed by RT-PCR at day 3, 14 and 21. At time points 3, 14 and 21 days total RNA was extracted by lysing the cells using TRIZOL reagent (Invitrogen) following