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Substrate fiber alignment mediates tendon cell response to inflammatory signaling

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ABSTRACT

Healthy tendon tissue features a highly aligned extracellular matrix that becomes disorganized with disease. Recent evidence suggests that inflammation coexists with early degenerative changes in tendon, and that crosstalk between immune-cells and tendon fibroblasts (TFs) can contribute to poor tissue healing. We hypothesized that a disorganized tissue architecture may predispose tendon cells to degenerative extracellular matrix remodeling pathways, particularly within a pro-inflammatory niche. This hypothesis was tested by analyzing human TFs cultured on electrospun polycaprolactone (PCL) mats with either highly aligned or randomly oriented fiber structures. We confirmed that fibroblast morphology, phenotype, and markers of matrix turnover could be significantly affected by matrix topography. More strikingly, the TF response to paracrine signals from polarized macrophages or by stimulation with pro-inflammatory cytokines featured significant downregulation of signaling related to extracellular synthesis, with significant concomitant upregulation of gene and protein expression of matrix degrading enzymes. Critically, this tendency towards degenerative re-regulation was exacerbated on randomly oriented PCL substrates. These novel findings indicate that highly aligned tendon cell scaffolds not only promote tendon matrix synthesis, but also play a previously unappreciated role in mitigating adverse resident fibroblast response within an inflammatory milieu.

Statement of Significance

Use of biomaterial scaffolds for tendon repair often results in tissue formation characteristic of scar tissue, rather than the highly aligned type-1 collagen matrix of healthy tendons. We hypothesized that non-optimal biomaterial surfaces may play a role in these outcomes, specifically randomly oriented biomaterial surfaces that unintentionally mimic structure of pathological tendon. We observed that disorganized scaffold surfaces do adversely affect early cell attachment and gene expression. We further identified that disorganized fiber surfaces can prime tendon cells toward pro-inflammatory signaling. These findings represent provocative evidence unstructured fiber surfaces may underlie inflammatory responses that drive aberrant collagen matrix turnover. This work could be highly relevant for the design of cell instructive biomaterial therapies that yield positive clinical outcomes.

1. Introduction

Tendons display a unique hierarchical organization of highly aligned type-1 collagen extracellular matrix (ECM) that enables efficient transfer of high mechanical forces from muscle to bone [1]. The physical demands on the tissue can overwhelm its intrinsic ability to self-repair, leading to an accumulation of damage and the onset of the pathological repair mechanisms that drive degenerative tendon disease [2]. Clinically termed tendinopathy, tendon degeneration is often painful, and accounts for approximately 30% of all orthopaedic consulations [3]. Tendinopathy is

Abbreviations: BGN, Biglycan; COL1, Collagen type 1; COL3, Collagen type 3; CCR7, C-C chemokine receptor type 7; DCN, Decorin; ECM, Extracellular matrix; IL1B, Interleukin-1beta; MKX, Mohawk; MMP1, Matrix metalloproteinase 1; MMP2, Matrix metalloproteinase 2; MMP3, Matrix metalloproteinase 3; MMP9, Matrix metalloproteinase 9; MMP13, Matrix metalloproteinase 13; MRC1, Mannose receptor C-type 1; PCL, Polycaprolactone; SCX, Scleraxis; TF, Tendon fibroblast; TGFB1, Transforming growth factor beta-1; TIMP1, tissue inhibitors of matrix metalloproteinase 1; TIMP2, tissue inhibitors of matrix metalloproteinase 2; TNF, Tumor necrosis factor alpha; TNMD, Tenomodulin.

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histologically characterized by structural derangement of the collagen matrix, hypercellularity, and vascular ingrowth.

Tendon fibroblasts (TFs) are the principal cell type in tendons, and often appear to be connected in a head-to-tail fashion along the length of parallel collagen fibers in healthy tissue [4]. TFs coordinate tissue adaptation and repair mechanisms regulated by various biochemical factors in synergy with a well-regulated balance between ECM synthesis and breakdown [5–7]. When this balance is disrupted, the collagen matrix composition becomes more heterogeneous and increasingly disordered [8]. Complex tissue changes follow, including cellular, vascular and neurological alterations [5,9,10]. Tendon injury was originally thought to be primarily associated with accumulation of mechanical damage and associated alterations of the tendon matrix [11,12]. This involves elevated matrix turnover with altered gene expression of the structural matrix proteins including *COL1*, *COL3*, *DCN* and *BGN* and certain proteases (e.g. metalloproteinases) that regulate mechanical and biological tissue homeostasis. The long-held misconception of tendinopathy as a "non-inflammatory" disease has only recently been exposed by evidence that early stage tendinopathy involves infiltration of immune cells, such as macrophages, mast cells, T lymphocytes and Natural killer cells [13–16]. These findings point to the coexistence of inflammation and degenerative changes in non-ruptured tendinopathic tendons, and open questions regarding the potential impact of immune-cells and their secreted factors on TFs. Among immune cells, macrophages are known to play a critical role in the coordination of the healing process in injured and diseased tissues [17], including tendon [15,18]. By extension, successful healing after implantation of a biomaterial graft or scaffold is also likely to require successful resolution of tissue repair mechanisms in an inflammatory milieu. Macrophages can play an active role in ECM turnover by secreting proteases, cytokines and other signaling molecules that may modulate the activity of TFs [19]. Although the spectrum of macrophage phenotypes and activities is broad, for conceptual simplicity, macrophages are often ascribed to one of two polarization profiles, namely a pro-inflammatory (M1-like) or an anti-inflammatory (M2-like) phenotype [19,20]. These two phenotypes are thought to represent the outer ends of a continuum of phenotypes ranging from M1 to M2, and have been subgrouped depending on their stimulation scenarios [20]. Initially, in response to tissue damage under inflammatory conditions, the M1 macrophages predominate and regulate processes such as phagocytosis and apoptosis [21,22]. M1 macrophages are characterized by the secretion of an array of pro-inflammatory cytokines including TNF and IL1B [19,20]. Furthermore, they have the ability to control ECM turnover by regulating the expression of various matrix metalloproteinases (MMPs) and tissue inhibitors of matrix metalloproteinases (TIMPs) [23]. In contrast, M2 macrophages are considered to suppress pro-inflammatory reaction and promote tissue homeostasis [24]. Cytokines-mediated regulation by differently polarized macrophages has been implicated in degenerative tendon disease (tendinopathy), regulating the interplay between TFs, immune cells and the ECM [25]. For example, inflammation-related nuclear translocation of the transcription factor NF-κB in tenocytes [26] has been attributed a critical role in the disease mechanism of tendinopathy [27].

Among many factors in the extracellular niche that regulate tendon cell behavior [28], extracellular matrix structure, particularly matrix fiber alignment, has emerged as a dominant factor in tendon fibroblast and tendon progenitor cell behavior [29–31]. These studies collectively indicate that highly aligned fibrous biomaterials tend to promote tenogenic cell differentiation, expression of tendon markers, and promotion of type-1 collagen synthesis and assembly. However, the converse hypotheses of these studies, that disorganized fibrous structures may promote aberrant matrix repair mechanisms, or even potentially confer sensitivity to pro-inflammatory signaling, have not been rigorously investigated. Such information would not only be relevant to the design of synthetic tendon biomaterial grafts, but also for a basic understanding of the mechanisms that may underlie tendon tissue pathologies.

We thus tested the hypothesis that tendon cells seeded on aligned biomaterial scaffolds would be less susceptible to a catabolic inflammatory stimulus than seeded on biomaterials presenting a more disorganized topography. We first assessed baseline behaviors of tendon cells on these two classes of materials in terms of tendon lineage markers, and markers of ECM turnover. We then probed how substrate driven cell morphology may predispose cell sensitivity to pro-inflammatory signaling. Our results reveal that the alignment of electro-spun polymer surfaces significantly affects tendon cell response to inflammatory signals such as those involved in early stages of tendon repair.

2. Materials and methods

2.1. Tissue collection and isolation of tendon fibroblasts

Fragments of healthy hamstring tendons were collected from male patients with a mean age of 23 ± 7 years, undergoing surgical reconstruction of the anterior cruciate ligament. Tissues were collected in accordance with granted ethical permission of the Canton of Zurich (permission number 2015-0089). Immediately upon collection, tendon tissues were immersed in Dulbecco's minimum essential medium (DMEM/F12 (Sigma)). Tendon fibroblasts (TFs) were then isolated following a previously described protocol [32]. Briefly, samples were stripped from surrounding tissue, cut into small pieces and dissolved in collagenase B (Roche). Next, the tendon fragments were digested for 6 h at 37 °C. The isolated cells were cultured in DMEM/F12 supplemented with 20% fetal bovine serum (FBS, Sigma), 1% penicillin-streptomycin (P/S, Sigma, Switzerland) and 1% amphotericin B (Gibco) for 7–12 days until 80% confluency was reached. At this time point, the cells were cryopreserved at −80 °C in 70% DMEM/F12, 20% FBS and 10% DMSO (Sigma). For all experiments TFs were thawed, expanded and deployed at P3.

2.2. Macrophage differentiation

The human monocytic leukemia THP-1 cell line (American Type Culture Collection) was cultivated in suspension in RPMI culture medium (Sigma-Aldrich Switzerland), supplemented with 10% FBS and 1% P/S at 37 °C in a humidified 5% CO_2 atmosphere and sub-cultured routinely before a cell density of 1×10^6 cells/ml was reached. Monocytes were differentiated towards M0 macrophages by stimulation with 100 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich, Switzerland) for 3 days, followed by 24 h cultivation with PMA-free cell culture medium [33,34]. Subsequently, M0 macrophages were chemically stimulated for 24 h with either 20 ng Interferon-γ (IFN-γ; Miltenyi Biotec) and 100 ng LPS (Lipopolysaccharide; Sigma) to obtain an M1 phenotype, or with 20 ng Interleukin-4 (IL-4; Miltenyi Biotec) to obtain an M2 phenotype. The chemical polarization towards the pro-inflammatory M1-like phenotype or the anti-inflammatory M2-like phenotype was validated using the specific surface markers CCR7 (M1) and MRC1 (M2). For experiments, M1 or M2 macrophages were seeded in Transwell™ Permeable supports (Corning, pore size: 0.4 μm) at a density of 1×10^5 cells/cm^2 and submerged in 2 ml of culture medium.

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