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Biochemical and biomechanical gradients for directed bone marrow stromal cell differentiation toward tendon and bone

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ABSTRACT

Substrates with mechanical property gradients and various extracellular matrix ligand loadings were evaluated for their ability to direct bone marrow stromal cell differentiation along osteogenic and tenogenic lineages. After verifying reproducible mechanical compliance characteristics of commercial hydrogel gradient substrates, substrates were functionalized with whole length fibronectin or collagen, both of which are found in skeletal structures and are relevant to cell-matrix signalling. Bone marrow stromal cells were seeded onto the substrates in growth media and cultured first to examine cell attachment and morphology, indicating higher levels of attachment on collagen substrates after 1 h, and increased spreading and organization trends after 24 h. Differentiation studies showed an increase in osteoblast differentiation on fibronectin substrates while collagen substrates lacked osteogenic differentiation. Osteogenic differentiation decreased on substrates of lower stiffness and lower ligand density. Molecular investigations revealed an increase in relevant signalling molecules for osteoblasts that were consistent with differentiation studies, but detected the presence of tenoblast markers on collagen substrates within a narrow range of stiffness. Our results indicate that mechanovariant substrates do hold promise as a culture platform for directed differentiation to tendon and bone by altering gene level expression of relevant signalling molecules. This study aids in understanding the molecular mechanisms that drive differentiation from substrate based cues, and could aid the design of therapeutic biomaterials at the transition from tendon to bone.

1. Introduction

The insertion site of both ligament and tendon into bone is subject to extreme mechanical demands and is prone to injury. Surgical methods are often used to reattach torn tendon to the bone, often delivering short term pain relief but remaining frequently plagued by poor clinical outcome, for instance with repair failure rates of massive rotator cuff tendon tears exceeding 80% [1]. This is generally attributed to insufficient restoration of native biochemical and mechanical properties at the insertion site. At the insertion site, a multi-tissue interface comprises a cellular and tissue transition from the tendon itself, to a fibrocartilage region, followed by bone. Cells within each zone actively secrete and assemble an extracellular matrix composed of collagens, proteoglycans, and calcified/noncalcified regions, with corresponding mechanical variations across the insertion [2]. The heterogeneous mechanical properties serve to minimize stress and allow load transfer from soft to hard tissue in a stable manner [3]. Given the inadequacy of current surgical approaches, biologic approaches to this problem have gained increasing attention in the laboratory and clinic [4], but progress has been limited by the required complexity of the tissue transition, the severe mechanical demands required of the final construct, and the diminished healing response in older patients.

Cell based therapies have been proposed as a potentially viable solution to restoring the tendon to bone transition, with promising therapeutic frameworks emerging that employ scaffold based approaches [5]; simply transplanting bone marrow mesenchymal stem cells seems not to improve the structure or strength of healing, and indicates that the repair site could lack necessary signals, both cellular and molecular, for appropriate differentiation [6]. To this end, tissue engineering approaches may offer potential for fabricating tissue structures by integrating pre-cellularized substrate–ligand systems that promote appropriate cell dynamics and

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tissue differentiation [7,8]. While homogeneous scaffold systems can be used to successfully engineer some tissues, this approach will likely not be feasible for the complex cell/tissue system required at the tendon-to-bone transition [3]. The ideal matrix to reestablish the tendon to bone transition would likely comprise a multiphase system that supports different cell/tissue types performing in concert. Two component systems combining a sponge and ceramic have proven somewhat successful after in vivo implantation, creating the desired transition between fibrocartilage and bone, while a triphasic system demonstrated feasibility of multi-tissue regeneration of all three tissue types in the composite scaffold [9,10]. While multiphase systems could employ differentiated cell phenotypes that are seeded in each phase prior to implantation, an attractive alternative would be to utilize mesenchymal stem cells (MSCs) or bone marrow stromal cells (BMSCs) [11–13]. Both are found in bone marrow, can proliferate rapidly, have the capacity to differentiate into a wide range of cellular phenotypes, and have been used to produce diverse tissues [14–17].

While stem cell differentiation has been extensively examined from a biochemical standpoint [18,19], examining MSC differentiation from a substrate/interface stance is still evolving, and in vitro approaches continue to yield important first insights. To this end, in vitro cell/tissue systems that integrate physical and molecular cues for early tissue development are often employed. Differentiation has recently been reported to be governed by interface properties such as surface/ligand chemistry and density. Ligand-presenting substrates have been examined in stem cell differentiation as a means to alter focal adhesion formation and subsequent signalling within the cell. Culturing stem cells on substrates with differential compliance functionalized with collagen, the substrate compliance alone was reported to be able direct cell commitment to specific lineages [20]. Substrate mechanics has been shown to affect cytoskeletal signalling elements that regulate differentiation and other behaviours like cell spreading [21], cell motility [22] and matrix assembly [23]. Such regulation relies both on occupancy of integrin receptors from a biochemical perspective, as well as from the mechanical perspective of tensional resistance at the cell substrate [24]. Recent evidence confirms that substrate stiffness alone may not be fully adequate to direct stem cell lineage specification, and that specific ligands together with substrate compliance regulate differentiation [25]. Thus mechanics based differentiation is likely to be critically interdependent with the extracellular matrix identity, and this may drive the mechanisms that mediate cell behaviour [26].

In the present work, we focus on BMSCs as a therapeutic cell source that can be induced to differentiate to different cell lineages, including musculoskeletal cells, depending on the ligand chemistry, density, and substrate stiffness. These variables regulate mitogen activated protein (MAP) kinase signalling which directly affects RhoA activity and gene level expression of transcription factors related to differentiation [26,27]. Our hypothesis was that using a substrate system with a gradient of mechanical compliances and strategic ligand choice, gene-level signalling could be modulated to create local regions of tenogenic and osteogenic differentiation on a single substrate. To explore cell sensitivity to substrate stiffness, we implemented hydrogels that featured a gradient of stiffness. The model proteins of this study were the widely characterized cell adhesion and signalling proteins, fibronectin and collagen type I (abbreviated as Fn and Col, respectively). Fibronectin is an extracellular matrix glycoprotein, playing a major role in cell dynamics like adhesion and migration during processes such as wound healing, development, and differentiation [28]. Collagen is the principal protein in connective tissues with multiple isoforms; type I is the most abundant [29] and is a key structural protein in both tendon and bone tissue [30]. We first examined early time point cell responses of attachment and spreading. Next, we examined the effect of ligand chemistry and substrate mechanics towards regulating stromal cell differentiation. Finally, we probed MAP kinase activity relative to bone and tendon cell differentiation.

2. Methods

2.1. Substrate characterization and preparation

NuPage 4–12% electrophoresis gel (Invitrogen) was used as the mechanovariant substrate. The gel is composed of 4–12% acrylamide and 3.8–5% bisacrylamide. The gel was sectioned into strips approximately 5 cm long and mechanical properties were determined with a material testing machine in tension, as well as compression using spherical indentation testing (Zwick 1456, Ulm, Germany). Elastic moduli determined by fitting indentation load relaxation data to an exponential decay function [31] were consistent with those derived from tensile experiments, but proved more reproducible and were used for quantitative characterization. Briefly, samples were mounted on a custom made platen and the sphere was brought in contact with the substrate. The relaxation profile was generated and fit to $P(t) = B_0 + \sum B_k e^{-t/\tau k}$ using a script in Matlab. Instantaneous and long-term elastic moduli (E_0 and E_∞) were calculated to determine the relative elastic and viscoelastic behaviour of the substrates. To verify that mechanical properties were consistently reproducible in these commercial substrates, measurements were taken every 0.5 cm from six independent samples. Three additional substrates were mechanically assessed after 14 days of cell culture to identify any long-term changes in substrate compliance.

Gels were washed in phosphate buffered saline (PBS) twice at 2 h intervals at room temperature before soaking overnight at 4 °C to remove excess acrylamide and sodium azide. The substrates were removed and strips that were approximately 3 cm in length were sectioned and placed in a six well plate (Falcon). The substrates were functionalized using procedures as described by others [32]. Substrates were overlaid with sulfo-SANPAH (Pierce Biotech), a light sensitive crosslinker, at 0.5 mg/ml in 50 mM HEPES buffer (Sigma). The substrates were then placed in a Stratalinker 2400 ultraviolet light crosslinker (Stratagene) for 10 min. The crosslinker was removed and the substrates were overlaid with fresh crosslinker and exposed to ultraviolet light. At this point the substrates were sterilized and then brought back to the sterile field for further processing.

To verify that uniform ligand distribution could be eventually be obtained using these substrates and the selected crosslinker, a series of substrates was functionalized with 200 nm fluorescent beads (Invitrogen) with surface amine groups. The beads were diluted to 1:10,000 in PBS and overlaid on substrates overnight at 4 °C. The substrates were washed and imaged to examine bead surface coverage on substrates. Once homogeneous distribution was verified, we functionalized substrates with our model ligands, human fibronectin or rat tail collagen type I (both from Sigma). Reactive substrates were overlaid with ligand overnight at 4 °C. The substrates were then washed three times in PBS and used immediately for cell culture and interface characterization experiments. To additionally verify uniform ligand loading on individual substrates, we performed immunofluoresence imaging with antibodies against the functionalizing ligands. Briefly, substrates were blocked with 1% bovine serum albumin (Sigma) for 1 h at 37 °C, washed, and incubated with rabbit anti-fibronectin or mouse anti-collagen primary antibody (Sigma) for the fibronectin or collagen functionalized substrates, respectively, overnight at 4 °C. The substrates were washed and incubated with the appropriate secondary antibody (Jackson Immunolabs) that was pre-labeled with FITC for 45 min at room temperature. Substrates were washed and images were acquired with a Nikon E600 upright epifluorescence microscope.

2.2. Cell culture

Human bone marrow stromal cells were a kind gift from Dr Simon Hoerstrup's lab (University of Zurich). The cells were first verified in that laboratory for multipotency in osteogenic, adipogenic, and chondrogenic induction assays [33]. After expansion and alliquotting for the present study, gene expression markers for multipotency (CD105, CD73, CD90 positive, CD34 negative) were confirmed using quantitative PCR as described below. Stromal cells were cultured in growth media, based in alpha MEM (Invitrogen) and supplemented with 10% fetal calf serum and 1% penicillin/streptomycin (both Invitrogen). Media was exchanged every three days and maintained at 37 °C with 5% CO_2. Cells were used just before confluence. A cell suspension was created from near-confluent flasks by washing cells with PBS, overlaying with trypsin and neutralizing with growth media.

2.3. Cell attachment

Cell attachment was analyzed by culturing 25,000 cells/cm^2 cells on functionalized substrates with 10 or 50 μg/ml of ligand (fibronectin or collagen type I) for 1 h at 37 °C. Substrates were washed with PBS to remove unbound cells, fixed with 3.7% formaldehyde (Sigma) for 15 min, washed with PBS three times, and overlaid with