Microtopographically patterned surfaces promote the alignment of tenocytes and extracellular collagen

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ABSTRACT

This paper investigates the role of microtopographical features on the cytomorphology, alignment, proliferation and gene expression of tenocytes. We made use of simple microfabrication approaches to create surfaces patterned with topographical features suitable for in vitro studies of tenocytes. These surfaces were composed of glass substrates patterned with polymeric ridges spaced from 50 to 250 μm apart. Our studies demonstrate that the microgrooves differentially impact tenocyte shape, alignment and matrix organization along the direction of grooves. Groove widths significantly influenced cellular alignment, with 50 μm grooved patterns affecting alignment most substantially. Polarized light microscopy demonstrated that mature collagen fibers were denser and more oriented within 50 μm patterns. None of the patterns had a significant effect on the expression of genes linked to proliferation or extracellular matrix synthesis, although time in culture profoundly influenced both gene groups. COMP mRNA expression was moderately increased in tenocytes seeded onto 250 μm grooves, but there was no overall beneficial phenotypic effect of aligned growth. The results of this study indicate that microtopography affects cell density and alignment of tenocytes and leads to the deposition of an aligned collagen matrix, but does not significantly impact matrix gene expression or cell phenotype. These outcomes provide insights into the biology of tendon regeneration, thus providing guidance in the design of clinical procedures for tendon repair.

1. Introduction

Tendons are highly specialized connective tissues that connect muscles to bone and transmit the tensile loads that move and stabilize joints. Tendon damage that occurs through repetitive strain or acute trauma is a major problem for the orthopedic biomedical community. Damaged tendons have a very limited capacity for regeneration, since these tissues are relatively avascular and sparsely populated with cells of low mitotic activity [1]. The ability of tendons to transmit tensile loads without structural failure is a consequence of the highly ordered arrangement of collagen fibers, aligned along the vector of primary load. When tendons are damaged, the resultant scar tissue is of inferior mechanical strength and elasticity. Conservative approaches to the treatment of tendon strain are currently focused on minimizing the acute inflammatory phase of repair and consequent scar deposition, with rehabilitation programs designed to optimize scar fiber orientation. Surgical treatments aim to stimulate vascular ingrowth and tissue repair,

reduce peritendinous adhesion formation and restore tendon–bone continuity [2]. These current therapies do not reliably restore an optimally organized tissue for tensile load-bearing. Tendon tissue engineering offers a promising alternative to regenerate damaged tendons. However, the factors that have the ability to stimulate tendon repair have not been identified.

Controlling cell–substrate interactions is critical to developing successful tissue engineering strategies. Recent advances in lithographic methods for microfabrication have facilitated substrate patterning and modification for cell studies. Researchers have studied the effect of surface topography [3,4], surface crystallinity [5], hydrophobicity [6], surface roughness [7] and chemical composition [8] on cellular responses. Cell substrate interactions have been studied in the context of patterned surface substrates for many cell types: fibroblasts [9–11], BHK cells [12,13], neuronal cells [14], Schwann cells [15–17], macrophages [18], epithelial cells [19], endothelial cells [20] and smooth muscle cells [21,22].

To date, the majority of studies addressing tendon tissue engineering have focused on developing biocompatible polymeric scaffolds for the directed migration of endogenous reparative cell populations [23,24] or ex vivo colonization of isolated cells prior to surgical implantation [25–27]. Mechanical loading is required

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for tenocyte homeostasis [28,29] and also impacts intrinsic tendon repair [30,31]. Biomechanical stimuli undoubtedly have an influence on the performance of any tissue-engineered tendon construct, but successful tendon regeneration requires a comprehensive understanding of the fundamental biological issues governing tenocyte growth and matrix orientation.

Tenocyte alignment and appropriate orientation of newly synthesized matrix are critical for tendon function [23,32]. However, the stimuli that influence the alignment of cells and the extent to which these factors affect tenocyte behavior are not yet well understood. In this work, we addressed the role of microtopographical factors on the alignment and growth of tenocytes. We fabricated substrates with groove widths ranging from 50 to 250 μm. These patterned substrates were used to investigate the influence of physical cues on the proliferation, cytomorphology, alignment and gene expression of tenocytes.

2. Materials and methods

2.1. Micropatterned substrate fabrication

Glass slides, 25 mm × 75 mm × 1 mm (Fisher Scientific, Pittsburgh, PA), were used as substrates for the fabrication of micropatterned structures. The slides were cleaned in piranha solution (H_2SO_4:H_2O_2 (30%) = 3:1) overnight, and subsequently rinsed with distilled water and dried in nitrogen. This was followed by sonication in isopropanol and acetone for 5 min each. After drying with nitrogen, the slides were exposed to oxygen plasma (100 torr, 100 W power) for 1 min. The micropatterned substrates were fabricated on the cleaned glass slides using standard photolithographic procedures. SU-8 5 photoresist (Microchem, Newton, MA) was spin-coated on the glass slides at 1000 rpm for 30 s. SU-8 is a biocompatible photoresist obtained by dissolving a polymeric epoxy resin (glycidyl ether of bisphenol A) in an organic solvent (gamma-butyrolactone) and adding a photoacid generator taken from the family of the triarylium–sulfonium salts [33]. The substrates were soft-baked at 65 °C for 2 min, followed by baking at 95 °C for 5 min. Transparency masks (CAD/Art services, Bandon, OR) of the pattern were placed on the glass substrates and exposed to UV light using a mask aligner (Suss Microtech MJB3) for 12 s (power = 334 MW). The substrates were then subjected to a two-step post-exposure bake: 1 min at 65 °C followed by 2 min at 95 °C. The patterns were developed in a propylene glycol monomethyl ether acetate solution (Sigma–Aldrich, St. Louis, MO) by dissolving the unexposed portions of the photoresist. After drying the substrates with nitrogen, they were exposed to UV light without a mask and hard baked at 150 °C on a hotplate overnight, to ensure complete polymerization of SU-8 and enhance its adhesion to the glass. The micropatterned slides were sterilized with hydrogen peroxide before use in cell culture experiments and were treated with oxygen plasma to promote cell adhesion. A schematic illustration of the micropatterned substrate is shown in Fig. 1a, along with a high-resolution of a 50 μm pattern (Fig. 1b). The grooves of the micropatterns were free from photoresist all the way down to the glass.

2.2. Tenocyte isolation and culture

Superficial digital flexor tendon specimens were collected from four adult horses that were euthanized for reasons not associated with musculoskeletal disease. These horses were euthanized in accordance with approved IACUC protocols by an intravenous overdose of a barbiturate anesthetic agent. The tendon specimens were diced and digested with trypsin–ethylenediaminetetraacetic acid for 1 h (Invitrogen, Carlsbad, CA) followed by overnight diges-
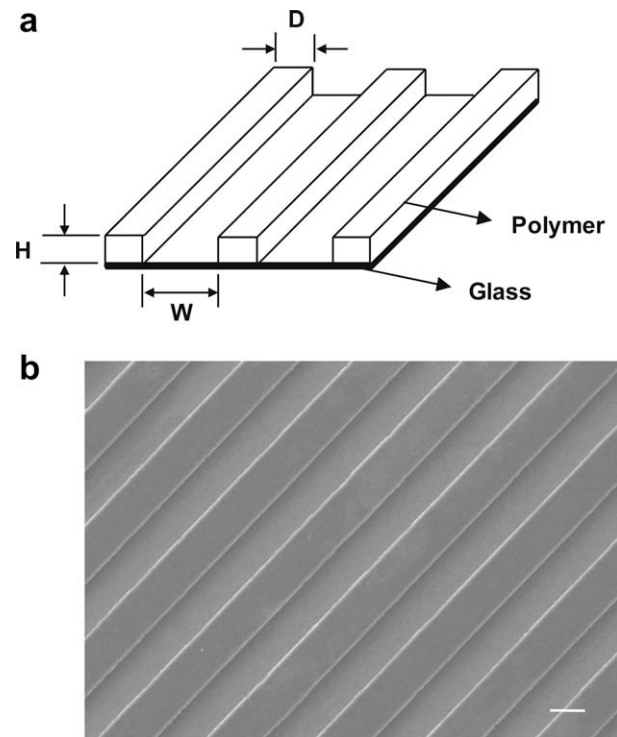

Fig. 1. (a) Schematic illustration of micropattterned substrates with critical dimensions: groove width (W), 50, 100 and 250 μm; ridge width (D), 50 μm; and ridge height (H), 15 μm. (b) Scanning electron micrograph image of a 50 μm grooved micropatterned substrate. Scale bar: 50 μm.

tion in 0.15% collagenase II (Worthington, Lakewood, NJ). The trypsin and collagenase digestions were carried out in a 37 °C shaking incubator. Following overnight incubation, the digest suspension was passed through a 40 μm pore-size filter to remove incompletely digested tissues. The released cells were pelleted by centrifugation at 390g for 10 min and washed twice in phosphate-buffered saline (PBS; HyClone, Logan, UT). The cells were counted using a hematocytometer and cryo-stored in freezing medium containing 50% high glucose Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT), 40% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA) and 10% dimethly-sulfoxide (Fisher Scientific, Rochester, NY) in liquid nitrogen.

2.3. Tenocyte seeding onto the patterned surfaces

The tenocytes were thawed, counted and seeded in 55 cm² culture dishes (Corning Incorporated, Corning, NY) at a density of 5×10^4 cells cm^{-2}. They were cultured in high glucose DMEM, supplemented with 10% fetal bovine serum, L-glutamine (2 mM) (Invitrogen, Carlsbad, CA), penicillin G sodium (100 U ml^{-1}), streptomycin (100 μg ml^{-1}) (Invitrogen, Carlsbad, CA), amphotericin B (2.5 μg ml^{-1}) (MP Biomedicals, Solon, OH) and ascorbic acid (50 μg ml^{-1}) (Wako, Richmond, VA) until confluence. The cells were then trypsinized and seeded onto the patterned surfaces at an initial density of 5×10^4 cells cm^{-2} in four-well rectangular dishes (Thermo Fisher Scientific, Rochester, NY) and cultured under 5% CO_2 at 37 °C for up to 72 h. The seeding density calculations were based on the surface areas of the microgroove floors available for cell attachment. Caution was taken to ensure that cells were not seeded in clumps thus ensuring uniform distribution. In each experiment, 10 replicates were seeded for each micropatterned substrate and time point: two patterns for confocal microscopy, two for SEM, two for optical microscopy and four patterns for RNA isolation.