Characterizing fibroblast migration on discrete collagen threads for applications in tissue regeneration

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Abstract: Collagen threads with mechanical properties and fibrillar substructure similar to native tissue have been synthesized for the repair of injured tendon and ligament. While these scaffolding materials have demonstrated the potential for inducing tissue regeneration, one limitation has been an insufficient rate of tissue ingrowth for complete regeneration. We hypothesize that the structural hierarchy and biochemical cues on the surfaces of these threads will enhance the rate of cell migration and ultimately the rate of new tissue ingrowth. We developed an *in vitro* assay to measure the effects of various collagen sources and crosslinking on the rate of fibroblast migration on the surfaces of collagen threads. Threads were suspended from elevated platforms and seeded with fibroblast-populated collagen lattices. Cell migration rates ranging from 0.75 to 1.25 mm/day were measured as the fibroblasts left the lattices and migrated onto various thread types. Threads self-assembled from type I collagen were found to have migration rates similar to native tendon threads while crosslinking by severe dehydration decreased the rate. This novel *in vitro* model system allows examination of cell migration from a wound margin onto biomaterials to determine the effects of various cell types, matrix materials, and surface biochemistries on cell–matrix interactions. Ultimately, this assay will allow us to identify design parameters that will be most effective for enhancing the rate of tissue ingrowth on fiber-based collagen scaffolds for soft tissue regeneration. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 55–62, 2004

Key words: fibroblast migration; collagen threads; tissue regeneration; cell–matrix interactions

INTRODUCTION

Fibrillar type I collagen is the basic structural element of soft connective tissues in the body. During development, tissue-specific cellular processes assemble type I collagen molecules into fibrillar elements several hundred nanometers in diameter, which then pack into larger scale fibril bundles and fascicles approaching 50–300 μm in diameter.[1] The structural and mechanical properties of these tissues can vary greatly depending on the collagen fiber hierarchy. In dense irregular connective tissues such as skin, dura mater, and cornea, arrays of collagen fibrils form sheet-like structures that can be modeled as woven or composite laminar materials.[2] In dense regular connective tissues such as tendons and ligaments, parallel arrays of fibrils and fascicles are uniaxially aligned into cable-like fibrillar structures that maintain the mechanical integrity and strength necessary for their load-bearing function.[2] Additionally, in response to soft tissue injuries, these fibrillar collagen scaffolds serve as templates that guide cell-mediated tissue synthesis, reorganization, and tissue regeneration.[3]

Many investigators have attempted to mimic these native tissue structures, developing collagen threads for the repair of soft tissue injuries. Collagen threads reconstituted from mature corium-derived insoluble type I collagen have been engineered into aligned, fibrous scaffolds to repair tendon and ligament injuries.[4–6] When implemented as tissue replacements in an animal model, these acellular scaffolds could promote neotissue ingrowth. However, nearly half of the collagen scaffold implants did not induce functional neotissue ingrowth. Furthermore, tissue ingrowth into these scaffolds was inconsistent and difficult to control.[5] Additionally, the implants failed to regain strengths comparable to the native tissue being replaced.[7–9] These findings suggest that the next generation of collagen scaffolds should possess structural hierarchy and biochemical cues that more closely mimic native tissue to enhance the rate of aligned tissue ingrowth, scaffold remodeling, and strength regeneration.[5,8,10] Towards this goal, investigators have developed a technique for producing self-assembled

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collagen threads from soluble collagen molecules that exhibit mechanical properties and fibrillar substructure that is comparable to native tendon tissue. These self-assembled threads also exhibit mechanical properties as well as fibrillar orientation and packing that is significantly greater than the collagen threads reconstituted from corium-derived insoluble type I collagen.[11]

To predict the rate of new tissue ingrowth onto these aligned fibrous scaffolds for soft tissue regeneration, several studies have focused on developing *in vitro* assays to measure contact-guided cell migration. Koob et al.[12] measured cell migration from tendon explants onto NDGA crosslinked collagen threads to determine material biocompatibility. Ricci et al.[13] developed a method for measuring cell colony growth rates on carbon fibers, but could not determine any variation in growth rates on different fiber types, except in the case where carbon fibers were compared with collagen-coated carbon fibers. Murray and Spector[14] measured non-oriented cell migration and proliferation from anterior cruciate ligament (ACL) explants into collagen–glycosaminoglycan (GAG) matrices in multiple dimensions. While each of these techniques provides some measure of tissue responses to implantable threads, there is presently no quantitative and definitive *in vitro* method for characterizing cellular functions such as fibroblast migration on the surfaces of collagen threads with precisely engineered surface topographies and extracellular matrix compositions.

It is our hypothesis that structural hierarchy and biochemical cues on the surfaces of self-assembled threads will enhance the rate of cell migration onto these scaffolds. To test our hypothesis, our laboratory developed an *in vitro* assay to measure cell migration from the edge of a model wound margin. In these initial experiments, we compared the effects of the two different collagen sources, as well as the effect of crosslinking, on the rate of fibroblast migration across the surfaces of collagen threads. Collagen threads were suspended across platforms in tissue culture dishes, and the platforms were seeded with fibroblasts-populated collagen gels. Fibroblast migration rates were determined by measuring the distances that cells traveled along the lengths of the various thread types as a function of time. The measured migration rates were found to increase on threads that were self-assembled from soluble collagen and decrease on threads crosslinked by severe dehydration. Ultimately, this novel *in vitro* model system may be a valuable tool for measuring contact-guided cell migration from a wound margin onto biomaterials with precisely engineered surface topographies and extracellular matrix compositions, and will elucidate the effects of various cell types, matrix materials, and surface biochemistries on cell–matrix interactions.

MATERIALS AND METHODS

Preparation of collagen solutions

Acid soluble type I collagen was obtained from rat tails as previously described.[15] Briefly, tendon fibers were dissected from Sprague-Dawley rats, rinsed in distilled water, and stirred in 1000 mL of 3% (vol/vol) acetic acid overnight. The supernatant was separated from the stock solution by centrifugation at 12,800*g* at 4°C for 2 h. The supernatant was precipitated with 200 mL of 30% NaCl (wt/vol) solution, and the pellet was collected by centrifugation at 4420*g* at 4°C for 30 min. The pellet was dissolved in 200 mL of 0.6% (vol/vol) acetic acid and dialyzed five times against 1.0 L of 1 m*M* HCl. The resulting collagen solution was lyophilized and stored at 4°C. The purity of the starting material was verified by SDS-PAGE. For collagen thread extrusion, a small quantity of type I collagen was dissolved in 5 m*M* HCl solution at a final concentration of 10 mg/ml and stored in syringes at 4°C.

Insoluble type I collagen from bovine Achilles tendon (Sigma, St. Louis, MO) was prepared as previously described.[6] For collagen thread extrusion, a 1% (wt/vol, 10 mg/mL) dispersion of insoluble type I collagen was prepared by blending 1.0 g of collagen material in 100 mL of 5 m*M* HCl for 10 min. The dispersion was degassed by centrifugation at 4420*g* at 4°C for 30 min and was stored in syringes at 4°C.

Collagen thread extrusion

Collagen threads were extruded from solutions of either soluble or insoluble type I collagen following a procedure similar to that described previously.[11] Briefly, collagen solutions were extruded through 0.38 mm inner diameter polyethylene tubing (Becton Dickinson, Inc., Franklin, NJ) using a syringe pump (KD Scientific, New Hope, PA) set at a flow rate of 0.7 mL/min. Threads were extruded into a bath of fiber formation buffer (pH 7.42, 135 m*M* NaCl, 30 m*M* TrizmaBase (Tris), and 5 m*M* $NaPO_4$ dibasic; Sigma, St. Louis, MO) maintained at 37°C overnight. The buffer was then replaced with a fiber incubation buffer (pH 7.42, 135 m*M* NaCl, 10 m*M* Tris, and 30 m*M* sodium phosphate dibasic, Sigma) that was maintained at 37°C for 24 h. The incubation buffer was then replaced with distilled water, and the threads were incubated at 37°C overnight. Finally, the threads were removed from the water bath, air dried, and stored at room temperature in a desiccator.

Half of the threads extruded from both soluble and insoluble collagen solutions were subsequently crosslinked by thermal dehydration (DHT) in which they were heated to 105°C under 100 mTorr vacuum for 24 h. These crosslinked threads were also stored at room temperature in a desiccator.

Native collagen thread controls were obtained by dissecting collagen fibers from rat tail tendons of Sprague-Dawley rats. Tendon fibers were split repeatedly along their axes under a microscope until the fibers were approximately 50 μm in diameter. These fibers were washed in distilled water, air dried while hanging, and stored in a desiccator.

Cell culture

For the cell migration assays, primary human dermal fibroblasts used in all experiments were isolated from neonatal foreskins and cultured in Dulbecco's Modified Eagles Medium (DMEM; Gibco BRL, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA) and penicillin/streptomycin (100 U/100 μg per mL; Gibco BRL). Briefly, sections of dermis from human neonatal foreskins were cut and placed onto scored areas of standard tissue culture dishes. The sections were allowed to dry for 30 min, and then were incubated in DMEM with 10% FBS and penicillin/streptomycin (100 U/100 μg per mL) at 37°C and 10% CO_2 until the culture dishes were confluent with fibroblasts. The fibroblasts were then removed by trypsin and expanded in culture. Passages 5–9 were used for all migration experiments.

Cell migration

Tissue culture systems for measuring the migration of cells onto threads were constructed using methods similar to those described previously.[13] Raised platforms of Thermanox® tissue culture plastic (5.5 × 15 mm) (Nalge Nunc, Rochester, NY) were elevated 1.5 mm above the surface of tissue culture dishes using molded silicone plugs of polydimethylsiloxane (PDMS; Sylgard 184, Dow Corning, Midland, MI) [Fig. 1(a,b)]. Threads were laid across the raised platforms, and the ends were attached to the tissue culture plates with Silastic® medical adhesive from silicone type A (Dow Corning, Midland, MI). Each experimental system contained 6 different thread types, including crosslinked (-X) and uncrosslinked collagen threads (self-assembled from acid-soluble type I collagen (SCT) or reconstituted from insoluble type I collagen (ICT)), native tendon threads from rat tails (NTT) for positive controls and Prolene® polypropylene suture (PPT) for negative controls (Ethicon, Somerville, NJ).

Migration tissue culture systems including all thread types were sterilized with 70% isopropanol 48 h prior to migration experiments. The systems were rehydrated in distilled water for 60 min, and then soaked in 70% isopropyl alcohol overnight. The alcohol was removed with 3 rinses of sterile distilled water for at least 60 min. The systems were then air dried in a laminar flow hood.

To seed cell migration assays, 0.5-mL aliquots of fibroblast-populated collagen lattice were placed onto each platform. These lattices were produced by mixing 3.0 mL of 5 × 10^5 cells/mL subconfluent human dermal fibroblasts (passages 5–9) with 2.4 mL of type I collagen (2.5 mg/mL) and 0.6 mL 5 × DMEM. After the gels were allowed to set at room temperature for 2 h, the migration plates were filled with 15 mL of DMEM supplemented with 10% FBS to a height just above the platforms and threads [Fig. 1(a,b)].

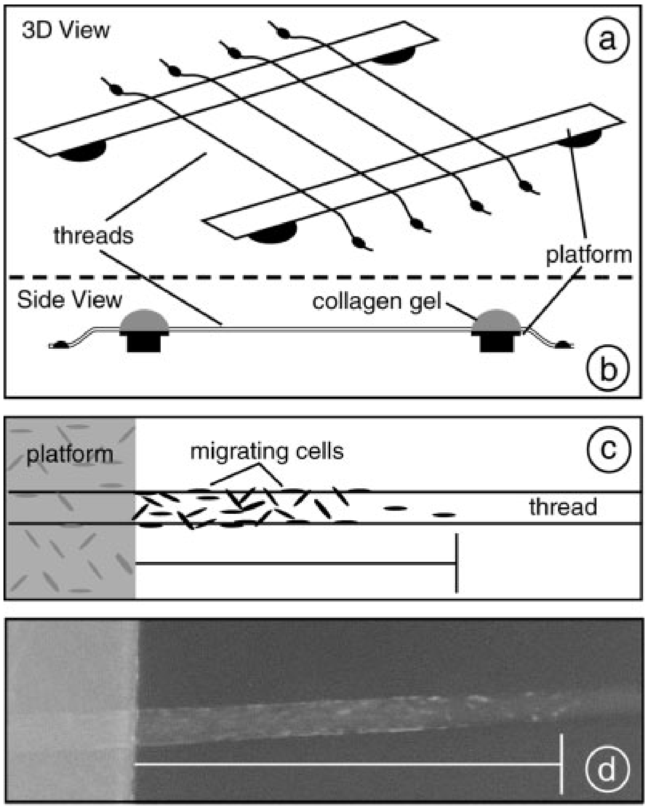

Figure 1. Fibroblast migration schematic. A schematic illustration showing the cell migration assay in both a 3D view (a) and a side view (b). A schematic illustration (c) and a digital image at day 4 (d) of cells fluorescently labeled with Fura-2 migrating from the platform onto a self-assembled collagen thread (SCT) as visualized under an inverted microscope. The measured distance to the furthest cell is illustrated by the horizontal bar in (c) and (d).

To measure cell migration on the collagen threads, fibroblasts were stained with a fluorescent intracellular Ca^{2+} probe, Fura-2 AM (Molecular Probes, Eugene, OR), every 24 h. One hundred microliters of 50 mM Fura-2 in dimethyl sulfoxide (DMSO; Sigma) was added to the cell culture media of the system and incubated for 30 min. The media was then replaced with 15 mL of fresh culture medium before the plates were viewed on a Nikon Eclipse TS100 inverted epifluorescent microscope coupled with a SpotRT CCD Camera (Diagnostic Instruments, Sterling Heights, MI) [Fig. 1(c,d)]. At each time point, measurements of distances migrated were made from the edge of the platform to the furthest fluorescently labeled cell using a calibrated reticle in the eye piece of the microscope.

Statistical analyses

All migration data was analyzed using SPSS for Windows (SPSS Inc., Chicago, IL). Data points of migration versus time were fit with a standard linear regression ($R^2 \geq 0.95$) to determine how well the average migration rates represented the data. The data points of average migration rate were then tested with a one-way ANOVA to determine a significant difference between the means. Post hoc comparisons between two sample groups were made with Tukey's honest significant difference (HSD) test with $p < 0.05$ indicating a significant difference between groups.

RESULTS

Fluorescent microscopy was used to measure cell migration distances from the edges of the cell-seeded platforms to the farthest fibroblast on various threads in order to quantitatively evaluate the capability of cells to interact with aligned scaffolding materials. No fluorescently labeled cells were visualized on any threads for the first 48 h [Fig. 2(a,d)]. However, 72 h after seeding the platforms with fibroblasts, cells began to migrate onto the threads [Fig. 2(b,e)]. Consequently, day 3 was considered the first time point for average migration rate (AMR) analyses. By day 4, while only a small number of cells were observed on the PPT threads, a large number of cells were observed on the SCT threads [Fig. 2(c,f)]. The cell density of migrating cells varied widely on different thread types; some threads (SCT, NTT) maintained a relatively high cell density and a front of migrating cells while other threads (ICT-X, PPT) were found to have limited cell density with no definitive front of migrating cells. Quantification of this phenomenon was estimated (data not shown), but distinct differences between thread groups were equivocal. In isolated cases, the threads detached from the plate and floated above the collagen gel on the platforms. These cases were considered to be invalid data points and, therefore, excluded from our analyses.

The total cell migration distance traveled each day was averaged for each type of thread and plotted as a function of time. These plots for each thread type were accurately modeled by standard linear regression ($R^2 > 0.95$), including both positive and negative controls (Fig. 3). Since the rate of migration is linear as a function of time, we averaged the distance traveled by the cells on days 3 through 8 to determine an average migration rate along each thread (AMR/thread) (Table I, column 2). These 5-day average values were each represented as a single data point for a particular thread extending from a particular platform. From all of these data points, averages and standard deviations were calculated (Table I, columns 3 and 4), and statistical comparisons between thread types were made using analysis of variance (ANOVA).

We compared the effects of the two different collagen sources, as well as the effect of crosslinking, on the average migration rates of fibroblasts on the surfaces of collagen threads. In these studies, the cell migration rates ranged from 0.75 to 1.25 mm/day. All of the collagen threads and the native tendon fibers were found to facilitate significantly greater AMRs than the PPT threads (Fig. 4). When cell migration rates on uncrosslinked threads were compared with DHT crosslinked threads, it was found that DHT crosslinking significantly decreased the rate of fibroblast migration on both self-assembled collagen threads and collagen threads reconstituted from insoluble collagen

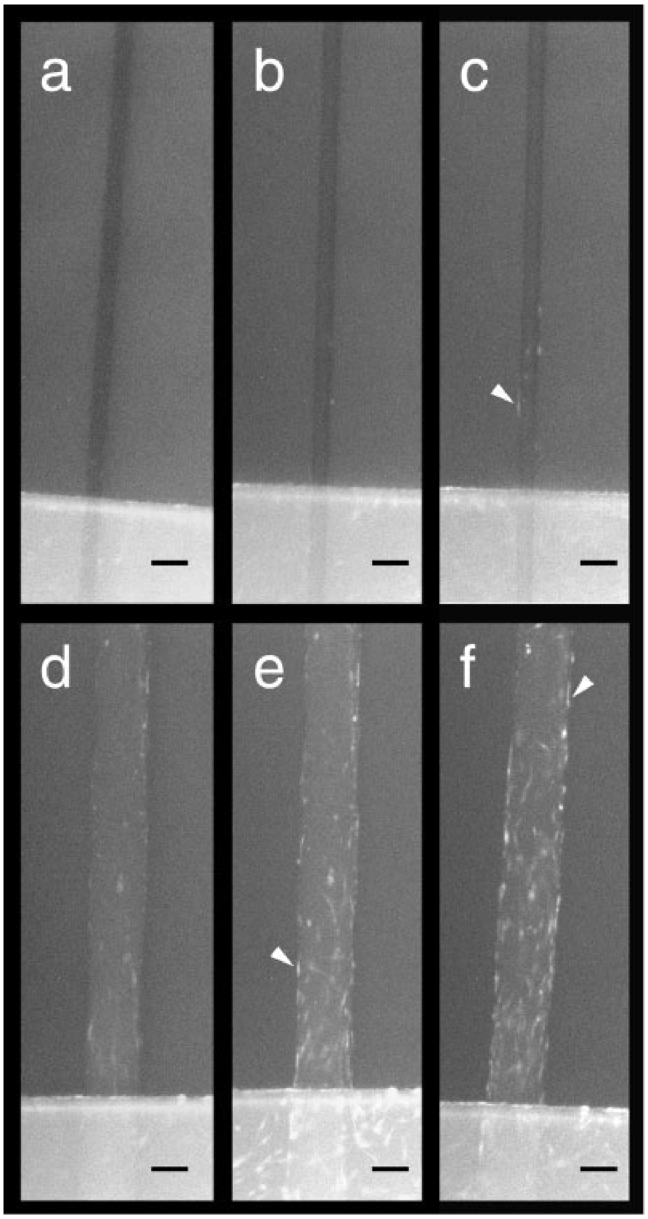

Figure 2. Fluorescent microscopy images of fibroblast migration. Inverted fluorescent microscopy images of Fura-2 labeled cells migrating onto a polypropylene thread (a–c) and a self-assembled collagen thread (SCT) (d–f) at day 2 (a,d), day 3 (b,e), and day 4 (c,f). Cells cannot be seen at day 2 on either thread. By day 4, a few initial cells can be seen leaving the platform onto the polypropylene thread (PPT) while many cells have migrated onto the self-assembled collagen thread (SCT). Examples of labeled cells are indicated with white arrowheads. Scale bars = 100μm.

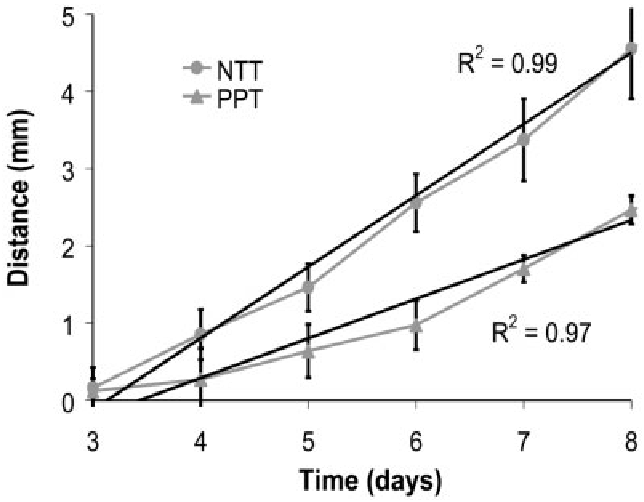

Figure 3. Average migration rates represent accurate metrics. Graph of migration distance versus time on control native tendon threads (NTT) and polypropylene threads (PPT) showing a strong fit with standard linear regression analysis. Average migration rates were determined to be accurate metrics of data obtained as a function of time.

(Fig. 5). However, SCT threads were found to promote a significantly greater migration rate than ICT threads under comparable crosslinking conditions (Fig. 6). Additionally, fibroblast migration rates on SCT threads were found to be comparable to the cell migration rates on NTT control threads (Fig. 7).

TABLE I
Average Migrahan Rate Means and Standard Deviations

	Average Migration Rate (days 3–8) (mm/d)		
Fiber Type	AMR/thread	Mean of Data Points	S.D.
NTT	1.21 0.98 1.04 1.04	1.06	0.10
SCT	1.10, 1.16 1.28, 1.34 1.06, 1.32 1.22	1.21	0.11
ICT	1.10, 0.73 1.22, 1.04 0.95, 0.79 1.04, 1.01	0.98	0.16
SCT-X	1.01, 0.79 0.77, 0.80 1.01, 0.98 0.67, 0.73	0.89	0.11
ICT-X	0.72, 0.82 0.81, 0.67 0.70, 0.98	0.76	0.10
PPT	0.30 0.61 0.37 0.67	0.49	0.18

[a] Data points of average migration rate (AMR) on each thread type between days 3–8, and the calculated mean and standard deviation (S.D.) of the sampled AMRs for like thread types.

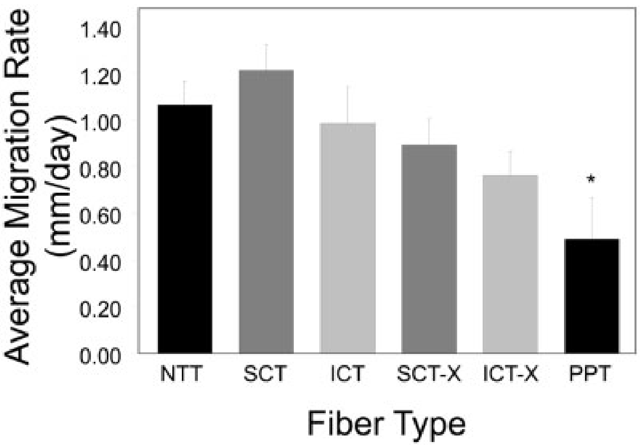

Figure 4. Average migration rates for all thread types are greater than negative controls (PPT). Average migration rates (AMR) for various thread types including controls. The polypropylene thread (PPT) negative control was found to have a significantly ($*p < 0.05$) slower AMR than all other thread types.

DISCUSSION

In this study, we describe a novel *in vitro* model system for quantitatively measuring cell migration onto threads. Cells in fibroblast-populated collagen

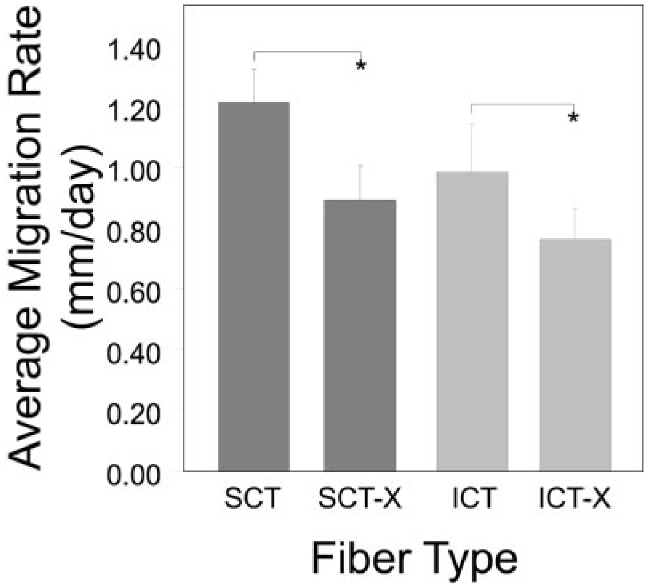

Figure 5. Crosslinking decreases migration rate. Average migration rates for self-assembled soluble collagen threads (SCT) and insoluble collagen threads (ICT). Crosslinked threads (-X) were found to have significantly ($*p < 0.05$) slower average migration rates than noncrosslinked threads of the same material.

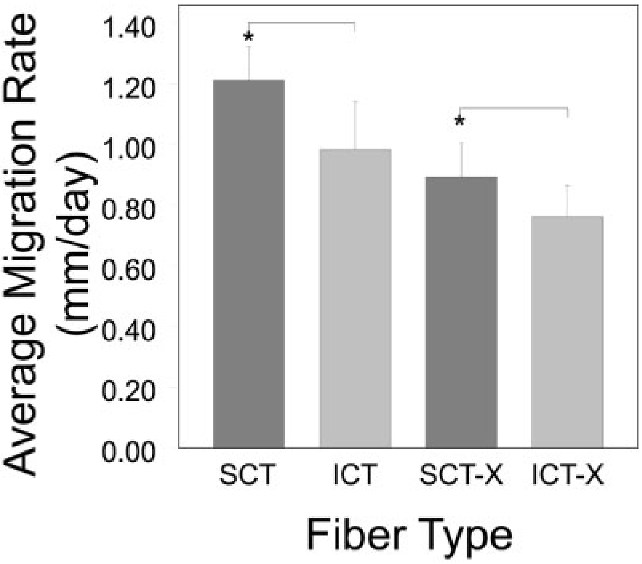

Figure 6. Self-assembled soluble collagen threads (SCT) promote faster migration than insoluble collagen threads (ICT). Average migration rates for self-assembled soluble collagen threads (SCT) and insoluble collagen threads, both crosslinked (-X) and uncrosslinked. The average migration rate for soluble threads was significantly (*$p < 0.05$) greater on soluble threads than threads made of insoluble collagen under the same crosslinking condition.

lattices migrated out of the matrix and onto the threads that were suspended across platforms submersed in culture medium. Since fibroblast-populated collagen lattices are commonly used as a model system to study wound healing,[16] the findings described in this study suggest that our experimental system can be used as an *in vitro* model to examine cell migration from a wound margin onto aligned fibrous scaffolds as a measure of cell-matrix interactions and new tissue ingrowth. The distances traveled per day were approximately constant on each thread type, providing a representative metric, average migration rate (AMR), for comparison between experimental conditions. The average migration rates of fibroblasts on collagen threads were measured to be between 0.75 and 1.25 mm/day, which correlates well to published data that determined the rate of tendon cell colony growth from explants onto collagen-coated Dacron fibers to be 1.53 ± 0.96 mm/day.[13] Unlike previous studies that could not determine significant differences in migration rates on various synthetic fiber types,[13] our method can accurately detect the fine variations between the measured migration rate on biological materials, which may be indicative of how well the scaffold will facilitate tissue ingrowth upon implantation.

The average fibroblast migration rate can be used to determine differences in how cells interact with a three-dimensional biologically relevant extracellular matrix composed of collagen from different sources and under varied crosslinking conditions. Since all of threads including common polypropylene suture threads used in this experiment are considered biocompatible, none totally inhibited cell migration. However, all threads composed of collagen showed a significantly greater average migration rate than synthetic threads. Since collagen is a ubiquitous structural element in soft connective tissues, as well as a fundamental component of wound healing processes, these findings are consistent with our initial hypothesis that the biochemical cues on the surfaces of the threads enhance the rate of cell migration onto fibrous scaffolds. These findings are also consistent with previous studies that showed tendon cell colonies grow faster on collagen-coated Dacron threads than on noncoated fibers.[13]

The results of this study using the novel *in vitro* method showed that the source of the collagen used to extrude the threads affected the average fibroblast migration rate. Threads self-assembled from solutions of soluble collagen molecules have previously been shown to possess improved mechanical properties with a higher density of aligned fibrils than threads extruded from insoluble collagen.[17] This increase in aligned fibril density in SCT threads more closely resembles the structure of native tendon threads. In this study, the SCT were shown to promote an increased average migration rate when compared to ICT threads and to have a migration rate that approxi-

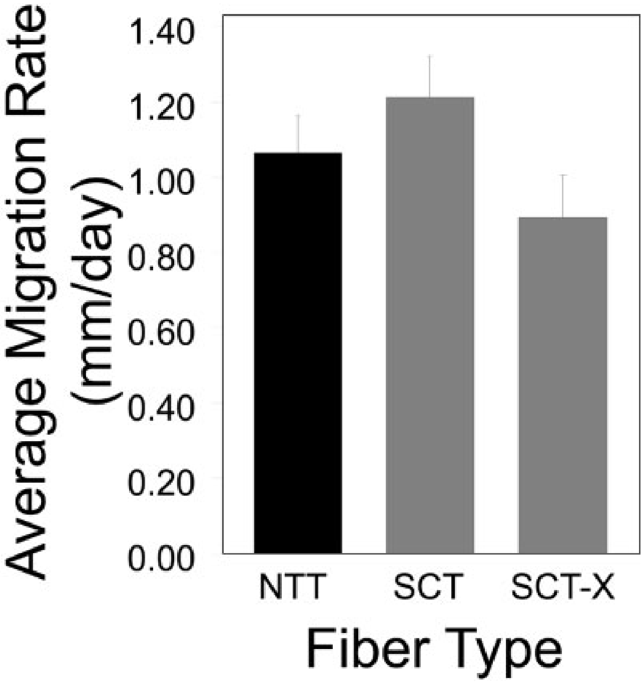

Figure 7. Self-assembled soluble collagen threads are similar to native tendon threads. Average migration rates for soluble collagen threads (SCT), crosslinked soluble collagen threads (-X), and native tendon threads (NTT). No significant difference (*$p < 0.05$) was found between native tendon threads (NTT) and self-assembled soluble collagen threads.

mately equals the migration rate of native NTT threads. This increase may be a direct result of the increased alignment of collagen fibrils in the SCT. SCT have been shown to possess increased fibrillar alignment following static axial stretching.[11] Similarly, contact guidance has been shown to affect cell behavior in oriented collagen gels.[18] Therefore, we hypothesize that increasing fibrillar alignment will increase the average cell migration rate by aligning the surface topography and altering extracellular contact guidance. Future tests are being conducted in our laboratory to determine whether fibrillar alignment affects the average migration rate. Using methods described in our previous work, various amounts of static axial stretching will be applied to SCT threads to impart varying amounts of fibrillar alignment.[11]

The results of this study suggest that DHT crosslinking may adversely effect cell-matrix interactions and the rate of tissue regeneration. Crosslinking of collagen using various techniques has been shown to increase the strength of collagen threads and decrease the rate of degradation. Crosslinking by severe dehydration, or DHT crosslinking, is a commonly used technique for physically crosslinking collagen without the addition of toxic chemicals that could be leached into the host tissue. While this technique has been shown to reduce collagen solubility, antigenicity, and degradation rate to stabilize the scaffolds upon implantation,[19,20] we believe that it may also mask integrin binding sites or surface receptors that mediate cell attachment and migration. The preliminary results of this study suggest that crosslinking decreases the average cell migration rate on collagen threads, regardless of the source of the material. This finding supports our hypothesis that DHT crosslinking inhibits cell-matrix interactions. The duration of DHT crosslinking (between 1–5 days) increases the quantity of crosslinks, which correlates with both an increase the mechanical strength and a decrease the solubility or rate of degradation.[21] Future tests will examine the effects of DHT crosslinking time on average cell migration rates to definitively determine whether an increase in crosslink density results in a decrease in cell migration.

The cell migration assay developed here may be useful in the future as an *in vitro* test of parameters that affect the regeneration of soft connective tissues including tendon and ligament tissue. The assay contains experimental parameters that can be altered for determination of differential characteristics including cell types, crosslinking methods, collagen substructure, or the microtopography on the surface of the threads. Ligaments have been found to have less healing capacity than tendons, resulting in the use of a patellar tendon as a common autograft for the repair of the anterior cruciate ligament in the knee.[22] One experiment might compare ligament fibroblasts with tendon fibroblasts to evaluate their relative abilities to migrate on aligned collagen threads, and therefore their ability to interact with the extracellular matrix during wound healing. The addition of wound healing cytokines may help elucidate the different intrinsic ability of cell types to regenerate in an *in vitro* wound healing model. Fibroblasts that are responsible for the wound healing capability of tendons are thought to originate in the epitenon, or the fascia surrounding native tendon.[12] Separate cultures of epitenon fibroblasts and internal tendon fibroblasts may help answer the question of the origin of the fibroblasts responsible for the increased wound healing capability seen in tendon. By keeping the cell type constant, crosslinking techniques, including both chemical and physical, may be evaluated for their suitability for engineering collagen thread constructs to optimize the rate of new tissue ingrowth.

Researchers have found that cells will migrate and proliferate on collagen threads under various crosslinking conditions *in vivo*,[12,23] but currently there is no quantitative deterministic methodology to characterize how cells migrate or the cellular interactions with these threads. Thus, up until now no *in vitro* comparison between crosslinking methods, collagen sources, surface biochemistries, or microtopographies has been available. In this study, we developed a sensitive technique to measure fibroblast migration on aligned fibrous scaffolds to elucidate the differences in cell matrix interactions and to predict the rate of tissue regeneration of grafts for soft tissue regeneration. Using this technique, we found that self-assembled collagen threads increase fibroblast migration rates while DHT crosslinking decreases fibroblast migration rates. The system also establishes a wound healing model of fibroblast migration from a wound margin. In the future, this *in vitro* assay will enable us to identify parameters to optimize the rate of tissue ingrowth for the development of constructs for soft tissue regeneration, as well as to determine fine variations in the cell-matrix interactions as a function of matrix materials and surface biochemistries.

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