

Cell orientation determines the alignment of cell-produced collagenous matrix

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Abstract

In healing ligaments and tendons, the cells are not aligned and collagen matrix is not organized as in normal tissues. In addition, the mechanical properties of the tissues are abnormal. We hypothesized that the lack of alignment of the collagen matrix results from random orientation of the cells seen in the healing area. To test this hypothesis, a novel *in vitro* model was used in which the orientation of cells could be controlled via microgrooves, and alignment of the collagen matrix formed by these cells could be easily observed. It is known that cells align uniformly along the direction of microgrooves; therefore MC3T3-E1 cells, which produce large amounts of collagen, were grown on silicone membranes with parallel microgrooves (10 μm wide \times 3 μm deep) in the surface. As a control, the same cells were also grown on smooth silicone membranes. Cells on both the microgrooved and smooth silicone surfaces produced a layer of readily visible collagen matrix. Immunohistochemical staining showed that the matrix consisted of abundant type I collagen. Polarized light microscopy of the collagen matrix revealed the collagen fibers to be parallel to the direction of the microgrooves, whereas the collagen matrix produced by the randomly oriented cells on the smooth membranes was disorganized. Thus, the results of this study suggest that the orientation of cells affects the organization of the collagenous matrix produced by the cells. The results also suggest that orienting cells along the longitudinal direction of healing ligaments and tendons may lead to the production of aligned collagenous matrix that more closely represents the uninjured state. This may enhance the mechanical properties of healing ligaments and tendons.

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1. Introduction

Ligaments and tendons are well-organized fibrous connective tissues. They are mainly composed of parallel collagen fibers interspersed with spindle-shaped fibroblasts that are aligned along the fibers in the longitudinal direction of the ligaments (Woo et al., 1994). However, after ligament injury, cells in the healing site are found to have no specific orientation. The resulting collagen matrix is also less organized, and this has been associated with the decrease in mechanical properties of the healing tissue (Frank et al., 1983; Weiss et al., 1991). *In vitro* studies have demonstrated that cells have the ability to reorient collagen fibers as a result of

contractility (Bell et al., 1979; Eastwood et al., 1998; Huang et al., 1993). Therefore, we hypothesize that the matrix organization is dependent on the orientation of the cells producing the matrix. In this study, a novel *in vitro* model was used to demonstrate the effect of cell orientation on the alignment of the collagen matrix that the cells produce by comparing elongated cells oriented parallel to one another to cells oriented randomly. Microgrooved membranes, which have been demonstrated to align a variety of different types of cells by contact guidance (Brunette and Chehroudi, 1999; Curtis and Wilkinson, 1997; Qu et al., 1996; van Kooten et al., 1998; Walboomers et al., 1999; Wang et al., 1995, 2000), were used to control cell orientation, whereas smooth silicone membranes were used as a control. MC3T3-E1 cells were chosen because they are an established cell line that is stable in culture from passage to passage (Quarles et al., 1992; Stein and Lian, 1993), and they can produce

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abundant collagenous matrix within a relatively short culture period (Hata et al., 1984). Unlike other cell-populated collagen gel models that use reconstituted collagen, this model provides the opportunity to study the effect of initial cell orientation on cellular collagen production and alignment in a model that more closely mimics tissue development and healing in vivo.

2. Materials and methods

Microgrooved silicone membranes were used as a substrate to grow cells in this study. Two silicone fluid components, 601A and 601B (Wacker Chemie, Munich, Germany), were added to a 60-mm Petri dish in a ratio of 10:1. The components were mixed well and then slowly poured onto a microgrooved silicon wafer, fabricated using lithographic and reactive ion etching techniques. After curing, the silicone membranes with microgrooves were removed. The dimensions and geometry of the microgrooves were measured with atomic force microscopy (AFM), and they were found to be rectangular in shape with a groove and ridge width of 10 μm and a depth of 3 μm (Fig. 1). The silicone compound was also poured over a plane of glass to make silicone membranes with smooth surfaces (i.e., without microgrooves), however, smooth silicone membranes from a commercial source (Specialty Manufacturing, Saginaw, MI) were used in most experiments to save time. Preliminary experiments determined that there was no apparent difference in cell morphology when the cells were grown on the two smooth silicone membranes. The microgrooved and smooth membranes were cut into small patches (approximately 1.5 cm \times 2 cm) for cell culture experiments, as described below.

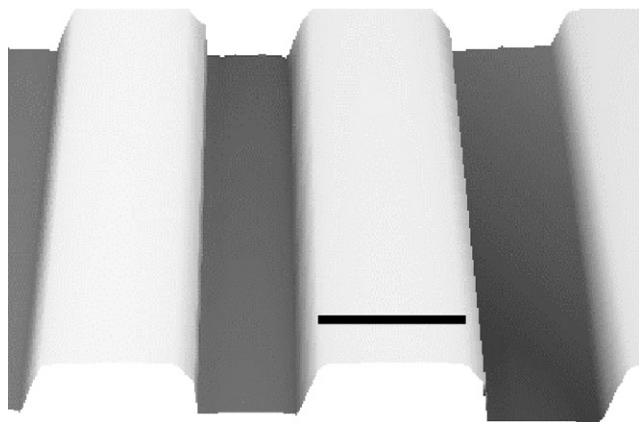


Fig. 1. Silicone microgrooves, imaged by AFM, were used as a substrate for growing cells. The grooves were rectangular in shape with a width of 10 μm , a depth of 3 μm , and spacing of 10 μm between the grooves. The light areas represent the top of the ridges, and the dark areas represent the bottom of the microgrooves (Bar: 10 μm).

Six microgrooved and smooth silicone patches were each placed in a 6-well plate and coated with 10 $\mu\text{g}/\text{ml}$ of ProNectin-F (BioSource International, Inc., Camarillo, CA) in phosphate buffered saline (PBS). ProNectin-F is a bioengineered protein that can promote attachment of cells to silicone surfaces (Wang et al., 2000). MC3T3-E1 cells were plated onto the ProNectin-F coated silicone patches at a density of 2×10^4 cells/ cm^2 . The cells were grown in α -MEM growth medium supplemented with 10% fetal bovine serum (FBS, Invitrogen, Carlsbad, CA), 100 units/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 5 mM β -glycerol phosphate, and 25 $\mu\text{g}/\text{ml}$ ascorbic acid (Sigma, St. Louis, MO). The cells were maintained at 37°C in a humidified atmosphere of 95% air and 5% carbon dioxide. The medium was changed every 3 days for 4 weeks. Phase contrast microphotographs were taken to examine the orientation of the cells on the microgrooved and smooth membranes.

Masson's Trichrome method (Gray, 1975) was used to stain the collagen matrix produced by the cells. With this method, collagens are stained in yellow and nuclei in black. All steps were performed at room temperature unless otherwise noted. Briefly, the matrix sample was first fixed with 3.7% paraformaldehyde in PBS for 10 min, dipped in 4% ferric ammonium sulfate solution at 50°C for 5 min, and rinsed with distilled water. The sample was then dipped in 1% hematoxylin solution at 50°C for 15 min, differentiated in 2% ferric ammonium solution at 50°C until only the nuclei were stained, and washed again with distilled water. Next, the sample was dipped in 1% phosphomolybdic acid for 5 min and rinsed with distilled water. It was then dipped in saturated metanil yellow and in 1% acetic acid for 5 min each. Finally, the matrix sample was rinsed with distilled water. Immediately after staining, each sample was observed on a light microscope and photographed with Kodak-100 slide films.

Polarized light microscopy was used to determine the orientation of collagen fibers in the cell-produced collagen matrices. The collagen matrix sample was placed on a histology slide and mounted on the planar stage of a polarized microscope (Nikon, Labophot-2). By adjusting the angle of the collagen sample relative to the analyzer, the alignment of the collagen fibers could be assessed. The collagen fiber alignment is related to the uniformity of the color displayed by the collagen fibers due to their polarizing effect (Whittaker and Canham, 1991). The polarized microscopy images of the collagen matrices from both microgrooved and smooth silicone membranes were photographed with Kodak-100 slide film.

An additional six microgrooved and smooth silicone membranes were used to determine the type of collagen that was present in the matrix by immunohistochemistry. The matrix sample was fixed with 3.7% paraformaldehyde, pretreated with 0.5% Triton-X100 for

6 min, and blocked with 5% bovine serum albumin for 2 h. Monoclonal anti-type I collagen antibodies (Sigma, St. Louis) were applied at a dilution ratio of 1:20 for 1 h and then washed with PBS. The FITC conjugated sheep anti-mouse antibodies (Sigma) were then applied at a dilution ratio of 1:100 for 1 h. The sample was washed extensively with PBS to remove excessive antibody, and then examined with fluorescence microscopy.

3. Results

Within hours of seeding the MC3T3-E1 cells onto the microgrooved membranes, they were oriented along the direction of the microgrooves and were uniformly elongated in shape. The same cells grown on the smooth surfaces were randomly oriented and tended to spread over the surface in all directions, unlike the uniform polarized shape of cells in the microgrooves (Fig. 2).

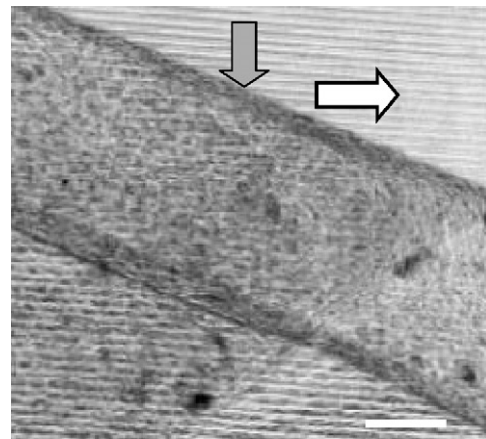
Phase contrast microscopy revealed that these cells on both the microgrooved and smooth silicone membranes produced a visible layer of matrix. In some of the samples, the matrix contracted due to the cells inside it, causing it to detach from the edges of the silicone membrane (Fig. 3). The matrix was readily stained by Masson's Trichrome, indicating that it was composed of collagens (Fig. 4). Masson's Trichrome also revealed that the cells (black staining represents nuclei) were generally elongated and parallel even after 4 weeks in culture. Immunohistochemistry further showed that the cell-produced collagen matrices on both microgrooved and smooth silicone membranes consisted of abundant type I collagen, shown with green due to the use of an FITC conjugated antibody (Fig. 5).

Most striking, however, was that the cells oriented along the microgrooves produced highly aligned collagen fibers that were also aligned in the direction of the microgrooves. Under a polarized light microscope, the collagen matrix appeared to be uniformly yellow, which indicates that the fibers were parallel to each other (Fig. 6A). In contrast, the collagen matrix produced by the cells on smooth surfaces diffracted the light into

multiple colors under polarized light, indicating that these collagen fibers produced by the cells on the smooth surfaces had no preferred alignment (Fig. 6B).

4. Discussion

In this study, a novel in vitro model was used to investigate the effect of cell orientation on the organization of the matrix the cells produce. This model uses a microgrooved silicone membrane to orient the cells parallel to one another, along the direction of the microgrooves, as demonstrated in previous studies (Brunette and Chehroudi, 1999; Curtis and Wilkinson, 1997; Qu et al., 1996; van Kooten et al., 1998; Walboomers et al., 1999; Wang et al., 1995, 2000). The novelty of this model is that collagen scaffold is



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Fig. 3. The matrix (darker area with solid arrow) shown here was formed by MC3T3-E1 cells grown on the microgrooved surface. As the matrix develops, the cells appear to migrate from silicone surface into the matrix. This is evident by the absence of cells on the microgrooved surface (lighter area with blank arrow) where the matrix has been contracted around the edges due to cell contraction (Bar: 120 μ m).

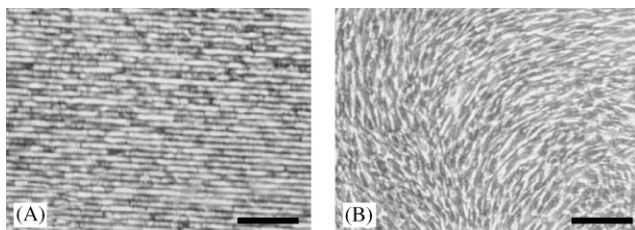


Fig. 2. Phase contrast microphotographs of MC3T3-E1 cells on the microgrooves (A) and smooth surfaces (B). The cells on the microgrooves were aligned along the direction of the microgrooves, that is, the horizontal direction, whereas the cells on the smooth surface were randomly oriented (Bar: 100 μ m).

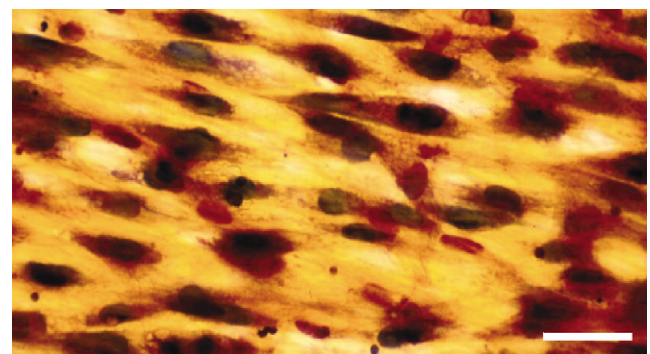


Fig. 4. Masson's Trichrome staining shows that the matrix was composed of abundant collagens (yellow). The cells (black spots represent nuclei) were embedded in the collagenous matrix (Bar: 50 μ m).

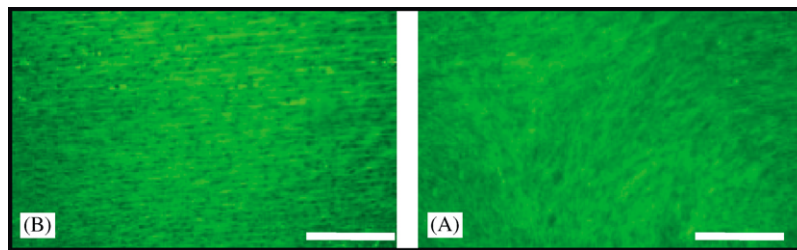


Fig. 5. Immunocytochemistry staining shows that the cell-produced collagen matrix in both microgrooved (A) and smooth (B) silicone membranes is composed of abundant type I collagen, which is shown with green because the FITC conjugated antibody was used. Note that omission of primary antibody to type I collagen resulted in no signal (Bar: 120 μm).

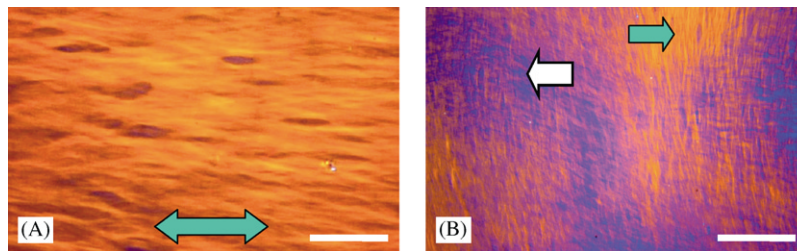


Fig. 6. The dense collagenous matrix produced by the cells in the microgrooves shows uniformly yellow under polarized microscope, indicating the collagenous fibers were aligned in the same direction as the microgrooves (green arrow, A). However, the collagen matrix produced by the cells in smooth surfaces shows multiple colors (B) and in this case, yellow (green arrow) and blue (white arrow). The multiple colors mean that the collagenous fibers had multiple orientations (Bar: 60 μm).

produced by the cells during the culture period, as opposed to the various studies that have been performed using reconstituted collagen from other sources (Bell et al., 1979; Bessea et al., 2002; Eastwood et al., 1998; Guido and Tranquillo, 1993; Huang et al., 1993; Okano and Matsuda, 1998; Seliktar et al., 2000). This renders an *in vitro* situation that is more like tissue development and healing since the cells are not just remodeling an existing matrix, but actually creating a new matrix with an organization dependent on the cell orientation. In addition, the use of polarized light gives the collagen alignment at a tissue level, as opposed to more labor intensive methods like electron microscopy which examine collagen alignment at the fiber level (Bessea et al., 2002).

The results of this study show that when cells were oriented along the direction of the microgrooves, they produced an aligned collagenous matrix; conversely, when they were randomly oriented, they produced a disorganized collagen matrix. This finding confirmed our hypothesis that cell orientation affects alignment of the cell-produced collagen matrix. The mechanisms for production of aligned collagen fibers by the aligned cells in this study may be due to cell contractility and motility (Hata et al., 1984; Wang et al., 2000). In culture, cells aligned in the direction of the microgrooves and were elongated in shape, and cells with this morphology have been shown to apply contraction forces along the cell's long axes (Wang et al., 2002). This directional contraction force may align collagen fibers in the same direction as that of the cells, as shown in previous studies with

exogenous collagen gels (Eastwood et al., 1998; Guido and Tranquillo, 1993; Huang et al., 1993). This increased alignment leads to an increased tissue stiffness which should increase the contractile force from the cell (Brown et al., 1998; Lo et al., 2000). Further, it has been shown that as the cells move and produce collagen, the collagen fibers orient in the direction of movement (Birk and Trelstad, 1986; Trelstad and Hayashi, 1979).

There are alternative or complementary mechanisms that could lead to the directional assembly and organization of the collagen fibers in this model. The elongated cell shape that develops as a result of the microgrooves could alter the type and concentration of integrins on the surface, influencing the charge of the surface and collagen binding (Friedl et al., 1998). It has been shown that $\beta 1$ integrins cluster at the binding sites between fibroblasts and collagen fibers, and are most prominent at the leading edge of the cell (Friedl et al., 1997; Lee and Loeser, 1999). Further studies are needed to elucidate the exact molecular mechanisms that are responsible for alignment of the collagen matrix in this study.

There are several limitations in this study. First, the development of the collagen matrix takes considerable time (4 weeks). Therefore, it is less convenient than models using exogenous collagen gels, although this model provides the opportunity to examine the directional assembly of the matrix over time in future studies. Second, only the presence of type I collagen was confirmed within the cell-produced matrix. Further studies are planned to determine how the cell shape

and orientation affect the type and amount of collagens produced by the cells. Finally, this study does not examine the effect of external mechanical loading on the matrix production by the cells and the remodeling of the matrix. This model, however, easily allows for the application of mechanical stretching as demonstrated in previous studies using similar models (Wang et al., 1995, 2000).

This study suggests that cell orientation affects collagenous matrix alignment. This supports the observation that randomly oriented cells in healing ligaments at the early healing stages are at least partially responsible for disorganized collagen matrix (Frank et al., 1983; Weiss et al., 1991). Mechanical load is also known to influence the organization of the healing ligaments (Gomez et al., 1991; Newton et al., 1995; Woo et al., 1982, 1987). Therefore, to restore the biochemical composition and biomechanical properties of healing ligaments and tendons to those of intact tissues, both cell orientation and mechanical loading of the healing ligaments should be considered. Future studies will evaluate the biochemical composition and mechanical properties of the cell-produced matrices to determine their potential to serve as a natural scaffolding material to enhance ligament and tendon healing.

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