Cell Motility and the Cytoskeleton 64:1–5 (2007)

Technique

A New Approach to Study Fibroblast Migration

Bhavani P. Thampatty and James H.-C. Wang*

MechanoBiology Laboratory, Departments of Orthopaedic Surgery, Bioengineering, and Mechanical Engineering, University of Pittsburgh, Pennsylvania

This paper presents a new approach to study cell migration. Human tendon fibroblasts were plated on silicone membranes coated with 10 μg/ml ProNectin-F. The silicone surfaces were micro-fabricated with parallel microgrooves, with 10 μm ridge and groove width, and 3 μm groove depth. Fibroblasts grown in the microgrooves had an elongated shape and oriented along the microgroove direction. They also moved along the same direction instead of "random walk" when cells migrate on smooth culture surfaces. In response to TGF-β1 (5 ng/ml) treatment, these fibroblasts on the microgrooved surfaces were differentiated into myofibroblasts, as judged by an elevated expression of α-smooth muscle actin (α-SMA), a specific marker for myofibroblasts. Moreover, these myofibroblasts were found to be ∼30% less motile compared to that of untreated fibroblasts. Thus, use of microgrooved surface may be an effective approach to detect difference in cell motility because cell migration on the microgrooved surface is one dimensional and hence easier to be quantified than two-dimensional random movement on conventional smooth culture surfaces. Cell Motil. Cytoskeleton 64:1–5, 2007.

Key words: fibroblast; migration; microgrooves; TGF-β

INTRODUCTION

Cell migration is a fundamental cellular process for normal development and homeostasis of tissue and organ. It is an essential characteristic of both physiological and pathological processes such as vascular and inflammatory diseases [Li et al., 2005]. It also plays a vital role in wound healing, tissue morphogenesis, angiogenesis, and metastasis [Webb et al., 2005]. Highly specialized cells such as fibroblasts are able to actively migrate within tissues. In wound healing, for example, fibroblasts migrate to the wound site for repairing damaged tissue and thereby heal the wound. During tissue healing, fibroblasts acquire contractile features by differentiation into myofibroblasts, which express a specific marker α-smooth muscle actin (α-SMA) [Jester et al., 1995]. TGF-β1 has been shown to be a potent inducer of α-SMA [Jester et al., 1999; Dalton et al., 2001]. TGF-β1 also has been shown to inhibit cell migration in monolayer wound assays [Tian and Phillips, 2003].

Because of the crucial role of cell migration in wound healing, several methods have been developed to

Contract grant sponsor: NIH; Contract grant number: AR049921; Contract grant sponsor: Arthritis Foundation.

*Correspondence to: James H.-C. Wang, Ph.D., MechanoBiology Laboratory, Biomedical Science Tower, E1640, 210 Lothrop Street, Pittsburgh, PA 15213. E-mail: wanghc@pitt.edu

Received 18 April 2006; Accepted 8 August 2006

Published online 19 September 2006 in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/cm.20166

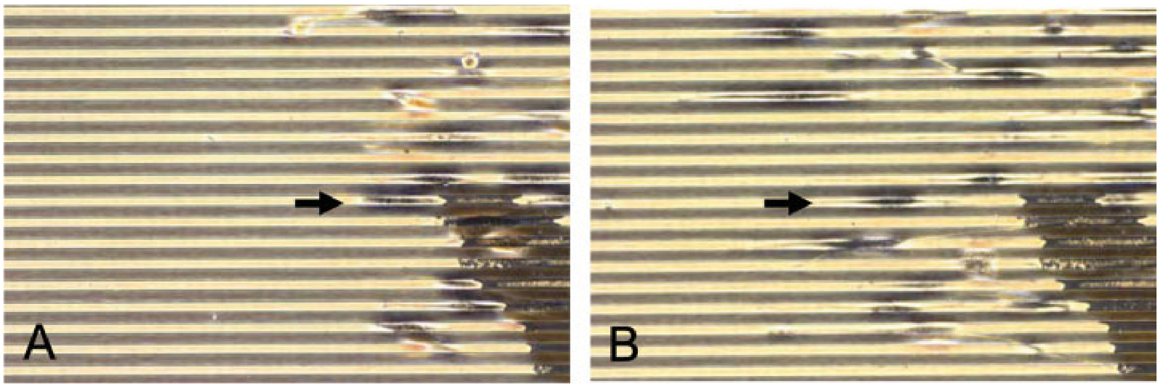

Fig. 1. Microgrooved silicone substrate with human tendon fibroblasts. Note that there are no cells on the left side of the image due to scratching. The black area in the bottom right corner is the ink dot used for a reference point to track cell movement. Also note that the arrows point to the same cell at the beginning of the experiment (**A**) and at the end of experiment, that is, 12 h later (**B**).

study cell migration [Kumar et al., 2005]. The cell monolayer wounding system is one of the most common methods to study cell migration in vitro. In these methods, cells are plated on a bare glass or plastic surface coated with a matrix protein, such as fibronectin or poly-L-lysine [Rodriguez et al., 2005]. On these smooth culture surfaces, cells do "random walk" and their movement is two-dimensional. As a result, the analysis of random movement is complicated and interpretation of cell migration changes due to experimental treatments is not straightforward. Therefore, it is necessary to develop a new method to study cell migration more effectively. The purpose of this study was then two-fold: (1) to develop a new technique for studying cell migration in one-dimension, (2) to apply such a new method to determine the motility of myofibroblasts compared to that of fibroblasts. Herein we report that microgrooved silicone surfaces can be used to align fibroblasts and control cell migration in such a way that their movement is along one direction. Using this new method, we show that myofibroblasts that are differentiated from fibroblasts in response to TGF-β1 treatment are less motile compared to non-treated fibroblasts.

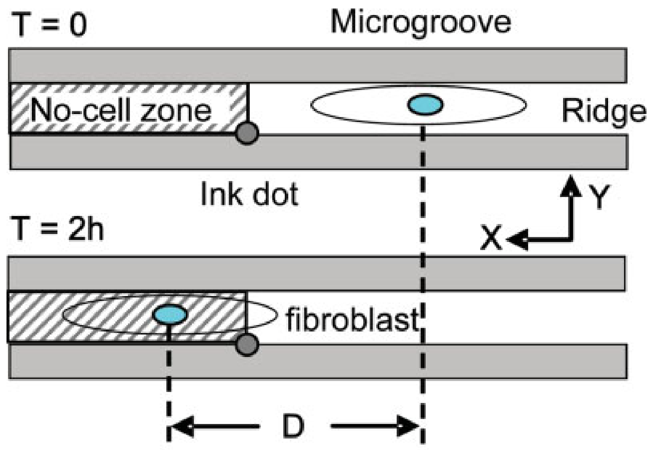

Fig. 2. An illustration of calculating cell migration on the microgrooved substrate. The distance of cell migration between two observation points (say, $T = 0$ and 2 h) were determined by image analysis of two images taken at the two time points. Note that the black dot at the corner of the no-cell zone was used as a reference point for measuring cell migration. Note that D is the distance a cell migrated between two time points.

MATERIALS AND METHODS

Preparation of Microgroove Surface

Microgrooved silicone membranes were made according to a procedure described previously [Wang et al., 2004, 2005]. Before use, the membranes were sterilized by autoclaving. The microgrooves were 10 μm in groove and ridge width, and 3 μm in groove depth. A syringe needle containing Sanford waterproof black India ink was used to apply dots on the microgrooved silicone membranes. These dots which were placed in a line perpendicular to the microgrooves were fixed to the membrane and thus were used as reference points (Fig. 1). Using these ink-dots as reference points, cell movements on the microgrooved membrane surface were tracked.

Cell Culture

Human patellar tendon fibroblasts (HPTFs) were isolated from tendon samples of a healthy male donor (age 21 years). The protocol for obtaining tendon samples was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (IRB #0407060). Briefly, tendon samples were washed thoroughly with PBS (Life Technologies, Rockville, MD), minced in Petri dishes aseptically, and transferred to 100

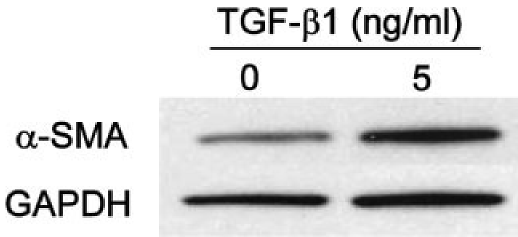

Fig. 3. A representative Western blot of α-SMA protein expression. TGF-β1 induced fibroblast differentiation into myofibroblasts, as evidenced by increased α-SMA expression levels. At least three separate experiments were performed and consistent results were obtained.

mm polystyrene Petri dishes. The cells were cultured in DMEM, containing 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 U/mL streptomycin (P/S; Life Technologies, Rockville, MD). The culture was maintained in a humidified atmosphere of 5% CO_2 at 37°C and subcultured up to six passages. Cell morphology and doubling time did not exhibit apparent changes between these passages.

To promote the adhesion of fibroblasts to silicone surfaces, ProNectin-F (Sigma, St. Louis, MO) at 10 μg/ml in PBS was used to coat microgrooved membranes in the six-well plates for 30 min. After washing the membranes with PBS twice, HPTFs in the sixth passage were plated on the microgrooved membranes at 10^4/cm^2 in DMEM containing 1% FBS in the first three wells of the six-well plate. These three wells were designated the control group. In the remaining three wells, the same concentration of cells was added with the same media, except containing 5.0 ng/ml of TGF-β1. The selection of the TGF-β1 dosage was based on previous studies which used dosages ranging from 1 to 10 ng/ml [Ellis and Schor, 1998; Bakin et al., 2002; Song et al., 2002]. This group of wells was designated as the experimental group. This six-well plate was incubated for 24 h at 37°C and 5% CO_2. After 24 h, a 2-ml pipette was used to make a rough scratch perpendicular to the microgrooves, thereby removing all cells along the scratch. The scratch was made adjacent to a row of ink dots. These detached cells were removed by replace the media with fresh ones.

Data Collection and Analysis

A CCD camera along with an image-grabbing software (Diagnostic Instruments, MI) were used to take microphotographs of at least 50 cells at 200× magnification in the no-cell zone. All digital photos were done in sets, with each set having at least one photo of an ink dot. A point on the ink dot was referred to as the origin (Fig. 2). Then, using the mobile base of the microscope, photos were taken at two varying points along the *y*-axis, and thus the *x*-coordinate of the origin remained constant. Images were taken every 2 h for 12 h (i.e., $T = 0$, $T = 2 \ldots T = 12$). Because of a low number of cells, the short period between time points, the high quality of phase contrast images, and the short distance of migration (<200 μm) along one direction (i.e. the microgroove direction), cells were easily distinguished at each of the time points (Fig. 1). Also note that to avoid the possible differential effects of ridges and grooves on cell migration, only cells on ridges of the microgrooved substrate were sampled for cell migration analysis.

At least 50 cells from each group were analyzed using Adobe Photoshop CS 8.0. Cells on the microgrooves only moved along the microgroove direction, which is defined as *x*-axis. The origin's *x*-coordinate was first recorded. Then, the *x*-coordinate of the center of a chosen cell, which is the average of the leftmost and rightmost points of the cell, was subtracted from the origin, which yield the distance the cell was away from the origin. Note that the distance was calculated using the groove width (10 μm) as a reference length.

Three migration measures, i.e., net movement, absolute net movement, and total movement, were used to quantify fibroblast migration on the microgrooved substrate. Net movement is the difference in distance of a cell from the origin at $T = 12$ and the distance of a cell from the origin at $T = 0$. The positive direction is designated as towards the scratch, while the negative direction is away from the scratch. Absolute net movement is calculated as the absolute value of the net movement, and thus direction of movement is disregarded. Total movement is defined as the absolute total distance traveled by the cell, i.e. the absolute value of the distance traveled between $T = 0$ and $T = 4$, plus the absolute value of the distance traveled between $T = 4$ and $T = 8$, and so on. All total movement values are always positive and greater than or equal to the respective net movement values. To examine trends in net movement, absolute net movement, and total movement, the median of the values was taken instead of the mean, which would be skewed because a few cells moved extremely long distances. Finally, for statistical data analysis, an unpaired *t* test was performed, with $P < 0.05$ considered significant.

Western Blot for α-SMA

Determination of α-smooth muscle actin (α-SMA) expression after TGF-β1 treatment was done following a standard Western blot procedure as described previously [Wang et al., 2004]. Briefly, the samples containing equal amounts of denatured proteins (10 μg) were fractionated by electrophoresis on 10% SDS-polyacrylamide gels, and the separated proteins were then transferred to a nitrocellulose membrane. The protein-bound membrane was incubated in a 5% non-fat milk/PBS-Tween 20 solution overnight to block nonspecific binding. After washing in PBS-Tween, the membrane was incubated

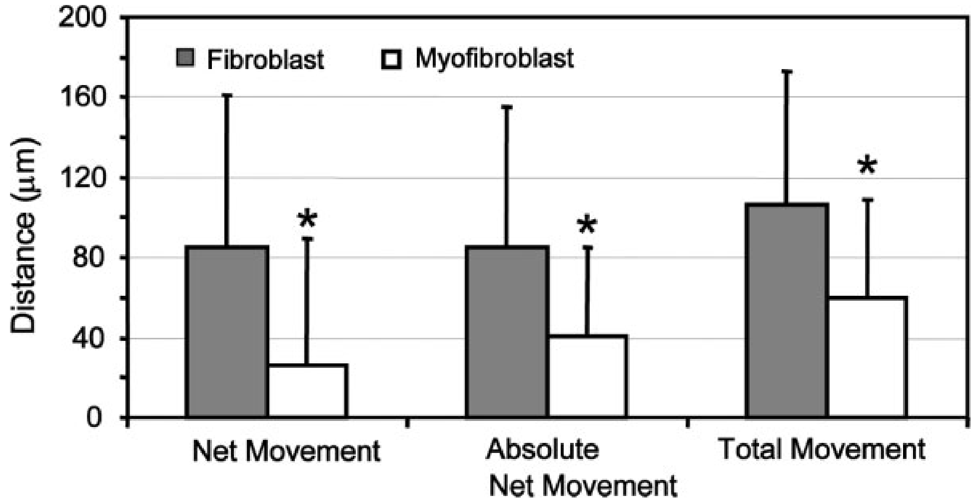

Fig. 4. The differential motility between myofibroblasts and fibroblasts. The chart shows that myofibroblasts moved slowed than fibroblasts, as determined by three measures for cell migration: the median net movement, absolute net movement, and total movement. (Total number of cells $n > 50$; $^*P < 0.05$).

for 1 h with α-SMA monoclonal antibody (Sigma-Aldrich, St. Louis, MO) followed by 1 h of incubation with goat anti-mouse IgG (Jackson Immunoresearch Lab, West Grove, PA). The membrane was washed three times with 0.1% PBS/Tween 20 for 15 min after each antibody application. The α-SMA protein on the nitrocellulose membrane was then detected with the ECL Plus detection system (Amersham Pharmacia Biotech, Piscataway, NJ) according to the manufacturer's protocol. The membrane was stripped in Restore Western Blot Stripping buffer (Pierce Chemicals, IL), and GAPDH was then probed for an internal control of equal protein loading.

RESULTS

The tendon fibroblasts in microgrooves became elongated and aligned along the microgroove direction (Fig. 1). The cells moved only in one dimension; that is, along the microgroove direction. Hence, the migration of each cell could be followed accurately. The same fibroblasts tracked at the beginning of experiment and 12 h later (Figs. 1A and 1B). The natural imperfections in the ink shape made it easy to keep track of the positions of nearby cells.

In addition, the tendon fibroblasts in the microgrooved surfaces increased α-SMA expression after treatment with 5 ng/ml TGF-β1 (Fig. 3). The result indicates that TGF-β1 treated fibroblasts in microgrooves became differentiated into myofibroblasts.

The migration of these differentiated myofibroblasts and fibroblasts on the microgrooved surfaces was tracked. It was found that myofibroblasts moved slower than fibroblasts (Fig. 4). Quantitatively, during 12 h of observation period, the median net movement of fibroblasts was 86 µm, whereas that of myofibroblasts was only 26 µm; that is, myofibroblasts that were differentiated from fibroblasts in response to 5 ng/ml TGF-β treatment, moved ~30% slower than fibroblasts. As mentioned earlier, these values were obtained by considering movement away from the scratch as negative. To disregard the direction of cell movement, median absolute net movement was calculated by taking the average of the absolute value of the net movement for each cell. Thus, when only the magnitude of the movement was considered, myofibroblasts and fibroblasts migrated 52 µm and 86 µm, respectively. Finally, the median total movement for myofibroblasts and fibroblasts was found to be 60 µm and 107 µm, respectively. Again, these two measures for cell migration also indicated that myofibroblasts moved slower than fibroblasts.

DISCUSSION

This study reports a new technique to determine fibroblast migration. We showed that when fibroblasts were grown on microgrooves surface, they became elongated and aligned with the microgroove direction, which mimicked the cell alignment in vivo. In addition, these cells also moved in one dimension. This is in contrast to the fact that cells on conventional smooth culture surfaces, the movement is random, not unidirectional, and hence difficult to measure the net movement of any selected cell. Thus, this new method for measuring cell migration is simpler in quantifying cell movement as well as interpretation of cell migration results compared to those using smooth surfaces. In addition, because microgrooved silicone membranes are highly elastic and transparent, they can be used to study cell motility under dynamic mechanical loading conditions.

Furthermore, cells on the microgrooved membranes increased α-SMA expression in response to TGF-β1 treatment, indicating that these fibroblasts differentiated into myofibroblasts. Induction of α-SMA by TGF-β1 has been reported previously in various cell types. Our results support that TGF-β regulates differentiation of fibroblasts into myofibroblasts [Cordeiro et al., 2000]. These differentiated myofibroblasts moved slower than fibroblasts, as shown consistently by three migration measures, i.e., the net movement, absolute net movement, and total movement (Fig. 4). The result is consistent with the previous observations that TGF-β (β1 and β2) is responsible for the inhibition of tendon cell migration [Conlon and Tomas, 2003]. Also, addition of TGF-β inhibited cell migration to the wound area in human dermal fibroblasts [Ohgoda et al., 1998] and in human renal epithelial cells [Tian and Phillips, 2003] where cell migration was examined using a monolayer wounding system.

Further studies should refine the new approach for cell migration study. First, the current technique may be combined with time-lapse video-microscopy so that cell migration can be continuously monitored. In addition, because microgroove dimension (groove width and depth) may affect cell orientation and motility [den Braber et al., 1996; Dalton et al., 2001; Yoshinari et al., 2003], the modification of current microgroove dimensions for studying motility of different types of cells may be needed. Finally, since TGF-β1 and TGF-β3 induce differential contraction in tendon fibroblasts [Campbell et al., 2004], their possible differential effects on fibroblast motility can be investigated using the current technique.

ACKNOWLEDGMENT

We thank Mr. Derrick Chu for assistance in performing cell migration experiment.

REFERENCES

Bakin AV, Rinehart C, Tomlinson AK, Arteaga CL. 2002. p38 mitogen-activated protein kinase is required for TGFβ-mediated fibroblastic transdifferentiation and cell migration. J Cell Sci 115:3193–3206.

Campbell BH, Agarwal C, Wang JH. 2004. TGF-beta1, TGF-beta3, and PGE(2) regulate contraction of human patellar tendon fibroblasts. Biomech Model Mechanobiol 2:239–245.

Conlon MA, Tomas FM. 2003. Inhibition of cell migration from tendon explants into fibrin clots by extracts derived from cheese whey is largely due to transforming growth factor-β. J Orthop Res 21:157–161.

Cordeiro MF, Bhattacharya SS, Schultz GS, Khaw PT. 2000. TGF-β1, -β2, and -β3 in vitro: Biphasic effects on Tenon's fibroblast contraction, proliferation, and migration. Invest Ophthalmol Vis Sci 41:756–763.

Dalton BA, Walboomers XF, Dziegielewski M, Evans MD, Taylor S, Jansen JA, Steele JG. 2001. Modulation of epithelial tissue and cell migration by microgrooves. J Biomed Mater Res 56:195–207.

den Braber ET, de Ruijter JE, Ginsel LA, von Recum AF, Jansen JA. 1996. Quantitative analysis of fibroblast morphology on microgrooved surfaces with various groove and ridge dimensions. Biomaterials 17:2037–2044.

Ellis IR, Schor SL. 1998. Differential motogenic and biosynthetic response of fetal and adult skin fibroblasts to TGF-β isoforms. Cytokine 10:281–289.

Jester JV, Petroll WM, Barry PA, Cavanagh HD. 1995. Expression of α-smooth muscle (α-SM) actin during corneal stromal wound healing. Invest Ophthalmol Vis Sci 36:809–819.

Jester JV, Huang J, Barry-Lane PA, Kao WW, Petroll WM, Cavanagh HD. 1999. Transforming growth factor(β)-mediated corneal myofibroblast differentiation requires actin and fibronectin assembly. Invest Ophthalmol Vis Sci 40:1959–1967.

Kumar G, Meng JJ, Ip W, Co CC, Ho CC. 2005. Cell motility assays on tissue culture dishes via non-invasive confinement and release of cells. Langmuir 21:9267–9273.

Li S, Guan JL, Chien S. 2005. Biochemistry and biomechanics of cell motility. Annu Rev Biomed Eng 7:105–150.

Ohgoda O, Sakai A, Koga H, Kanai K, Miyazaki T, Niwano Y. 1998. Fibroblast-migration in a wound model of ascorbic acid-supplemented three-dimensional culture system: The effects of cytokines and malotilate, a new wound healing stimulant, on cell-migration. J Dermatol Sci 17:123–131.

Rodriguez LG, Wu X, Guan JL. 2005. Wound-healing assay. Methods Mol Biol 294:23–29.

Song QH, Klepeis VE, Nugent MA, Trinkaus-Randall V. 2002. TGF-β1 regulates TGF-β1 and FGF-2 mRNA expression during fibroblast wound healing. Mol Pathol 55:164–176.

Tian YC, Phillips AO. 2003. TGF-β1-mediated inhibition of HK-2 cell migration. J Am Soc Nephrol 14:631–640.

Wang JH, Yang G, Li Z, Shen W. 2004. Fibroblast responses to cyclic mechanical stretching depend on cell orientation to the stretching direction. J Biomech 37:573–576.

Wang JH, Yang G, Li Z. 2005. Controlling cell responses to cyclic mechanical stretching. Ann Biomed Eng 33:337–342.

Webb DJ, Zhang H, Horwitz AF. 2005. Cell migration: An overview. Methods Mol Biol 294:3–11.

Yoshinari M, Matsuzaka K, Inoue T, Oda Y, Shimono M. 2003. Effects of multigrooved surfaces on fibroblast behavior. J Biomed Mater Res A 65:359–368.