

Short Communications

Fibroblast responses to cyclic mechanical stretching depend on cell orientation to the stretching direction

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Abstract

Fibroblasts in intact tendons align with stretching direction, but they tend to orient randomly in healing tendons. Therefore, a question arises: Do fibroblast responses to mechanical stretching depend on their orientation? To address this question, human patellar tendon fibroblasts were grown in custom-made silicone dishes that possess microgrooved culture surfaces. The direction of the microgrooves was either parallel or normal to the direction of cyclic uniaxial stretching. Fibroblasts grown in these microgrooves had a polar morphology and oriented along the direction of the microgrooves regardless of the stretching conditions. Tendon fibroblasts expressed higher levels of α -smooth muscle actin when they were oriented parallel to the stretching direction than when they were oriented normal to the stretching direction. Also, cyclic stretching of the fibroblasts perpendicular to their orientation induced a higher activity level of secretory phospholipase A₂ compared with stretching of the cells parallel to their orientation. Thus, these results show that fibroblast responses to mechanical stretching depend on cell orientation to the stretching direction.

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1. Introduction

In vitro model systems are widely used to investigate the effects of mechanical forces on cells. Typically, cells are grown on deformable materials with smooth culture surfaces and subjected to cyclic mechanical stretching. It has been shown that cyclic stretching of cells induces various biological responses, including remodeling of actin cytoskeleton (Dartsch and Hammerle, 1986; Wang et al., 1995) and changes in cell proliferation (Buckley et al., 1988; Neidlinger-Wilke et al., 1994), gene expression and protein synthesis (Carver et al., 1991; Leung et al., 1977). It has also been noted that under cyclic uniaxial stretching, many types of cells (e.g., fibroblasts and smooth muscle cells) change their orientation (Buck, 1980; Dartsch and Hammerle, 1986; Wang et al., 1995). Fibroblasts in intact tendons, however, align with the stretching direction, but they tend to orient randomly in healing tendons. So the

question then arose: Do fibroblast responses depend on cell orientation with respect to the stretching direction?

Under uniaxial stretching, cells with different orientations will be subjected to different axial substrate strains that act along the cells' long axes (Wang et al., 1995), and also the axial substrate strains determine actin cytoskeletal remodeling (Wang, 2000). Thus, we hypothesized that cell responses to uniaxial mechanical stretching would depend on cell orientation with respect to the stretching direction. To test this hypothesis, human patellar tendon fibroblasts were grown in custom-made silicone dishes containing microgrooved culture surfaces. The direction of the microgrooves was either parallel or perpendicular to the dish's long axis, where the long axis was also the stretching direction. Cell alignment in the microgrooves was examined with light microscopy, and the level of α -smooth muscle actin (α -SMA) expression was measured by Western blot. In addition, the activity levels of secretory PLA₂ (sPLA₂), an enzyme that mediates cellular production of eicosanoids, were measured with ELISA. The α -SMA and sPLA₂ were chosen because they are often expressed in fibroblasts in response to environmental stimuli (de Beer

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et al., 1997; Hinz et al., 2001a). The results of this study showed that both α -SMA expression and sPLA₂ activity levels of the human patellar tendon fibroblasts were dependent on cell orientation with respect to the stretching direction.

2. Materials and methods

Silicone dishes were used to grow and apply cyclic mechanical stretching to human tendon fibroblasts. Silicone is transparent, elastic, and non-toxic to cultured cells, and it has successfully been used in previous cell stretching experiments (Wang et al., 1995; Wang and Grood, 2000). The silicone dishes were fabricated by mixing two liquid components, 601A and 601B (Wacker Silicones Corporation, Adrian, MI) in a ratio of 10:1. The silicone mixture was subjected to a vacuum to extract air and then poured slowly into a multiple-dish mold made of acrylic (Plexiglass). After removing the dishes from the mold, silicone dishes with smooth culture surfaces were obtained.

To obtain silicone dishes with microgrooved culture surfaces, silicon wafers containing parallel microgrooves on their surfaces were first made using standard lithographic and reactive ion etching techniques. The wafers were then used to mold microgrooved silicone membranes. The obtained microgrooved membranes were bonded, using silicone adhesive (Dow Corning, MI), to the bottoms of silicone dishes whose smooth culture surfaces had been removed. The microgrooved culture surface of the dish was 3×6 cm, with the microgrooves either parallel or perpendicular to the dish's long axis, which was also the stretching direction (Fig. 1a). The microgrooves were rectangular in profile, with $10 \mu\text{m}$ ridge and groove width, and $3 \mu\text{m}$ groove depth (Fig. 1b).

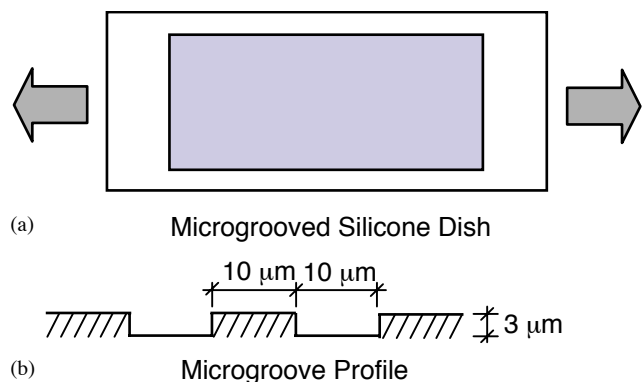


Fig. 1. Silicone dishes were used for growing and stretching tendon fibroblasts. The dishes had microgrooved surfaces with a culture area of 3×6 cm (a). The microgrooves had a rectangular profile (b) and were either aligned parallel or perpendicular to the stretching direction (arrows).

The silicone dishes were coated with $10 \mu\text{g}/\text{ml}$ ProNectin (BioSource International, Inc., Camarillo, CA) to promote cell attachment. Human patellar tendon fibroblasts were isolated from tendon pieces trimmed from patellar tendon autografts of healthy donors (Male, 20 and 40 years old) undergoing ACL reconstruction. The protocol for collecting human tendon samples was approved by the Institutional Review Board of the University of Pittsburgh Medical Center (IRB # 0108109). The fibroblasts were subcultured up to seven times, and no apparent changes in cell morphology or doubling time were noted.

The fibroblasts were plated at a density of 2×10^4 cells/cm² to silicone dishes with microgrooves either parallel or perpendicular to the stretching direction. The cells were grown in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin (Invitrogen, CA). After incubation for 24 h, the silicone dishes were mounted on a custom-made stretching apparatus, with a similar mechanism described previously (Neidlinger-Wilke et al., 2001). An 8% stretching magnitude was applied at 0.5 Hz for up to 72 h. In order to verify cell alignment before and after stretching, digital microphotographs were taken of fibroblasts in the microgrooved silicone dishes. In separate experiments, after 8% stretching at 0.5 Hz for 4 h, followed by 4 h of rest, the stretched as well as non-stretched fibroblasts were lysed to collect cellular proteins. A $20 \mu\text{g}$ protein sample was loaded into a 10% polyacrylamide gel for electrophoresis, and separated proteins were then transferred to a nitrocellulose membrane. After blocking with 5% non-fat milk in 0.1% PBS-Tween 20, the membrane was then incubated with a mouse anti-human monoclonal antibody to α -SMA (Sigma, St. Louis, MO) with a ratio of 1:1500 in PBS-0.1% Tween 20 containing 1% non-fat milk at room temperature for 2 h. Then, the membrane was washed extensively and incubated for 2 h with the secondary antibody, which was a goat anti-mouse IgG conjugated with horseradish peroxidase (1:5000 dilution ratio; Jackson Immunoresearch Lab, Inc., West Grove, PA). Finally, actin bands on the membrane were detected using the ECL system (Amersham, Piscataway, NJ). In addition, the medium was collected and used to measure sPLA₂ activity levels using an ELISA kit (R&D Systems, Minneapolis, MN) according to the protocol provided by the manufacturer. ANOVA was used for statistical analysis, with a significance level set at 0.05.

3. Results

Regardless of whether the microgrooves of the dish were oriented parallel or perpendicular to the stretching direction, the tendon fibroblasts aligned with the microgrooves (Fig. 2). Also, α -SMA expression levels

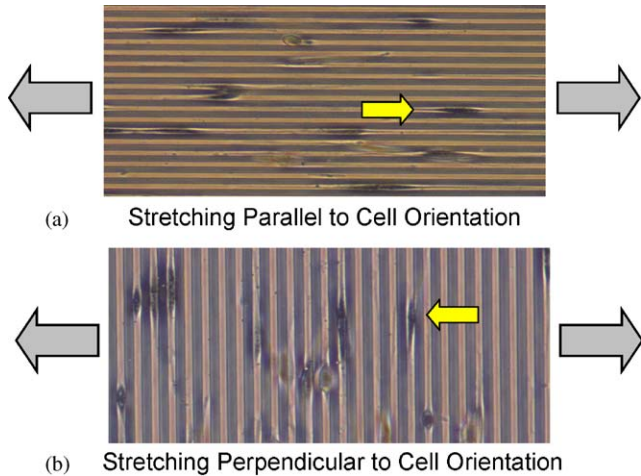


Fig. 2. Human tendon fibroblasts were aligned in the microgrooved surfaces without stretching and the cell alignment was maintained with 8% cyclic mechanical stretching for up to 72 h, regardless of whether the fibroblasts were oriented parallel (a) or perpendicular to the stretching direction (b). Note that the yellow arrows point to fibroblasts that were oriented parallel (a) or perpendicular (b) to the stretching direction.

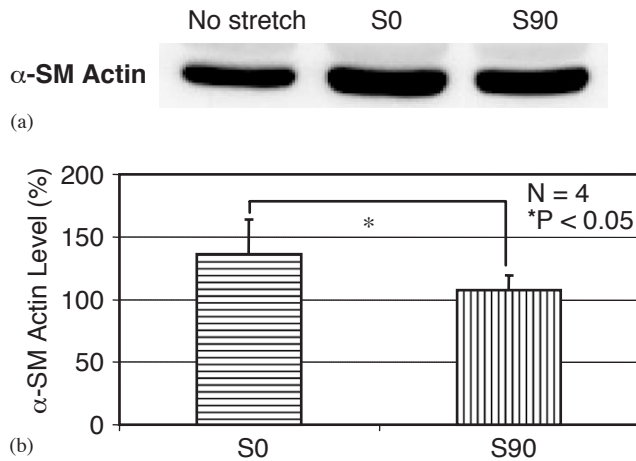


Fig. 3. A representative result of α -SMA expression levels of human tendon fibroblasts on the microgrooved surfaces (a). When cyclic uniaxial stretching was applied parallel to the cell orientation, the α -SMA expression levels were significantly increased. However, when the cells were stretched perpendicular to their orientation, the α -SMA expression levels were not significantly increased compared with that of non-stretched cells (b). Note that the α -SMA expression levels of both groups (i.e., S0 and S90) were normalized with respect to non-stretched group. (S0: stretching parallel to cell orientation; S90: stretching perpendicular to cell orientation).

of the fibroblasts stretched parallel to cell orientation were significantly increased compared to that of the cells stretched perpendicular to their orientation (Fig. 3).

Furthermore, sPLA₂ activity level of stretched fibroblasts was significantly increased, compared with that of non-stretched cells. The level of the increase in the sPLA₂ activity, however, was significantly higher in fibroblasts that were stretched perpendicular to their

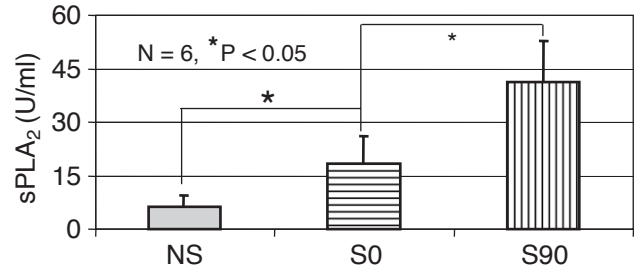


Fig. 4. The sPLA₂ activity levels of human tendon fibroblasts in response to cyclic uniaxial stretching. Stretching of the fibroblasts either parallel (S0) or perpendicular (S90) to their orientation increased sPLA₂ activity levels compared with that of non-stretched cells, but perpendicular stretching induced a significantly higher level of sPLA₂ activity than parallel stretching. Note that the same numbers of fibroblasts were present in the three experimental groups (i.e., non-stretched, S0, and S90).

orientation, as compared with those cells stretched parallel to cell orientation (Fig. 4).

4. Discussion

Despite the same uniaxial stretching conditions being applied to the microgroove substrate, α -SMA expression and sPLA₂ activity levels of tendon fibroblasts differed, depending on the orientation of the cells with respect to the stretching direction (Figs. 3 and 4). Therefore, it is likely that fibroblast responses to mechanical stretching in general depend on orientation of the cells to the stretching direction. Thus, to define fibroblast responses to cyclic uniaxial stretching, it is necessary to control cell orientation.

This study showed that mechanical stretching increases α -SMA expression levels. Since α -SMA is a marker of myofibroblasts (Hinz et al., 2001a), the result suggests that mechanical stretching can induce differentiation of fibroblasts into myofibroblasts, an interesting hypothesis that requires further testing. The result of α -SMA expression is consistent with previous findings that mechanical loading increases α -SMA expression in smooth muscle cells (Tock et al., 2003) and that mechanical tension in granulation tissues controls myofibroblast differentiation (Hinz et al., 2001b). In addition, the finding that cyclic mechanical stretching induces higher levels of sPLA₂ is also consistent with the previous finding that mechanical stretching of avian skeletal muscle cells enhances PLA₂ activity (Vandenburgh et al., 1993). The increased sPLA₂ activity levels suggests that mechanical stretching of tendon fibroblasts may induce tendon inflammation when cells in wound sites are stretched perpendicular to their long axes, or their orientation.

Note that cyclic stretching of fibroblasts on the microgrooved surfaces did not change their alignment, regardless of whether the cells were aligned parallel or

perpendicular to the stretching direction. Cell contact guidance is thought to be responsible for the cell alignment on the microgrooved surfaces (Dunn and Heath, 1976) or on the strands of fibronectin (Mudera et al., 2000). This cell alignment in microgrooved surfaces is in contrast to cell reorientation on smooth culture surfaces in response to uniaxial stretching (Buck, 1980; Buckley et al., 1988; Dartsch and Hammerle, 1986; Wang and Grood, 2000). Therefore, a microgrooved silicone substrate is effective in applying defined substrate strains relative to the long axis of the cell. This makes it possible to study mechanobiological responses of the cells (e.g., tendon or ligament fibroblasts) more closely.

The mechanism responsible for the cell-orientation-dependent fibroblast response remains unclear. However, it is clear that although the same stretching (i.e., 8% uniaxial stretching) was applied to microgrooved surfaces, the tendon fibroblasts were subjected to two different loading conditions with respect to their long axes (i.e., tension vs. compression). Therefore, the resulting different cell deformations may activate different mechano-transduction mechanisms, such as integrin-cytoskeleton (Ingber, 1991), mechanically sensitive ion channels, and load-conformation sensitive receptors (Banes et al., 1995). The different mechano-transduction mechanisms may be responsible for the different fibroblast responses observed in this study.

A limitation of this study was that it examined only two cellular responses (α -SMA expression and sPLA₂ activity levels) and one type of cell under a single stretching condition. Future studies should determine global changes in phenotypic expression of tendon fibroblasts or other types of cells under various stretching conditions using gene and protein profiling technologies (Pelech et al., 2003; Schild and Trueb, 2002).

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References

Banes, A.J., Tsuzaki, M., Yamamoto, J., Fischer, T., Brigman, B., Brown, T., Miller, L., 1995. Mechanoreception at the cellular level: the detection, interpretation, and diversity of responses to mechanical signals. *Biochemistry and Cell Biology* 73, 349–365.

Buck, R.C., 1980. Reorientation response of cells to repeated stretch and recoil of the substratum. *Experimental Cell Research* 127, 470–474.

Buckley, M.J., Banes, A.J., Levin, L.G., Sumpio, B.E., Sato, M., Jordan, R., Gilbert, J., Link, G.W., Tran Son Tay, R., 1988.

Osteoblasts increase their rate of division and align in response to cyclic, mechanical tension in vitro. *Bone and Mineral* 4, 225–236.

Carver, W., Nagpal, M.L., Nachtigal, M., Borg, T.K., Terracio, L., 1991. Collagen expression in mechanically stimulated cardiac fibroblasts. *Circulation Research* 69, 116–122.

Dartsch, P.C., Hammerle, H., 1986. Orientation response of arterial smooth muscle cells to mechanical stimulation. *European Journal of Cell Biology* 41, 339–346.

de Beer, F.C., de Beer, M.C., van der Westhuyzen, D.R., Castellani, L.W., Lusic, A.J., Swanson, M.E., Grass, D.S., 1997. Secretory non-pancreatic phospholipase A2: influence on lipoprotein metabolism. *Journal of Lipid Research* 38, 2232–2239.

Dunn, G.A., Heath, J.P., 1976. A new hypothesis of contact guidance in tissue cells. *Experimental Cell Research* 101, 1–14.

Hinz, B., Celetta, G., Tomasek, J.J., Gabbiani, G., Chaponnier, C., 2001a. Alpha-smooth muscle actin expression upregulates fibroblast contractile activity. *Molecular Biology of the Cell* 12, 2730–2741.

Hinz, B., Mastrangelo, D., Iselin, C.E., Chaponnier, C., Gabbiani, G., 2001b. Mechanical tension controls granulation tissue contractile activity and myofibroblast differentiation. *American Journal of Pathology* 159, 1009–1020.

Ingber, D., 1991. Integrins as mechanochemical transducers (Review). *Current Opinion in Cell Biology* 3, 841–848.

Leung, D.Y., Glagov, S., Mathews, M.B., 1977. A new in vitro system for studying cell response to mechanical stimulation. Different effects of cyclic stretching and agitation on smooth muscle cell biosynthesis. *Experimental Cell Research* 109, 285–298.

Mudera, V.C., Pleass, R., Eastwood, M., Tarnuzzer, R., Schultz, G., Khaw, P., McGrouther, D.A., Brown, R.A., 2000. Molecular responses of human dermal fibroblasts to dual cues: contact guidance and mechanical load. *Cell Motility and the Cytoskeleton* 45, 1–9.

Neidlinger-Wilke, C., Wilke, H.J., Claes, L., 1994. Cyclic stretching of human osteoblasts affects proliferation and metabolism: a new experimental method and its application. *Journal of Orthopaedic Research* 12, 70–78.

Neidlinger-Wilke, C., Grood, E.S., Wang, J.-C., Brand, R.A., Claes, L., 2001. Cell alignment is induced by cyclic changes in cell length: studies of cells grown in cyclically stretched substrates. *Journal of Orthopaedic Research* 19, 286–293.

Pelech, S., Sutter, C., Zhang, H., 2003. Kinetworks protein kinase multiblot analysis. *Methods in Molecular Biology* 218, 99–111.

Schild, C., Trueb, B., 2002. Mechanical stress is required for high-level expression of connective tissue growth factor. *Experimental Cell Research* 274, 83–91.

Tock, J., Van Putten, V., Stenmark, K.R., Nemenoff, R.A., 2003. Induction of SM-alpha-actin expression by mechanical strain in adult vascular smooth muscle cells is mediated through activation of JNK and p38 MAP kinase. *Biochemical and Biophysical Research Communications* 301, 1116–1121.

Vandenburgh, H.H., Shansky, J., Karlisch, P., Soleressi, R.L., 1993. Mechanical stimulation of skeletal muscle generates lipid-related second messengers by phospholipase activation. *Journal of Cell Physiology* 155, 63–71.

Wang, H., Ip, W., Boissy, R., Grood, E.S., 1995. Cell orientation response to cyclically deformed substrates: experimental validation of a cell model. *Journal of Biomechanics* 28, 1543–1552.

Wang, J.H., 2000. Substrate deformation determines actin cytoskeleton reorganization: A mathematical modeling and experimental study. *Journal of Theoretical Biology* 202, 33–41.

Wang, J.H., Grood, E.S., 2000. The strain magnitude and contact guidance determine orientation response of fibroblasts to cyclic substrate strains. *Connective Tissue Research* 41, 29–36.