

Treadmill Running Exercise Results in the Presence of Numerous Myofibroblasts in Mouse Patellar Tendons

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ABSTRACT: Mechanical loading is known to alter tendon structure, but its cellular mechanisms are unclear. This study aimed to determine the effect of mechanical loading on tendon cells in vivo. C57BL/6J female mice were used in a treadmill running study. The treadmill running protocol consisted of treadmill training for 1 week, followed by sustained moderate running at 13 m/min for 50 min/day, 5 days/week, for 3 weeks. Immunohistochemical staining of tendon sections of mice after treadmill running revealed that numerous cells in the tendon section expressed α -SMA, whereas in the tendon sections of control mice, only a few cells exhibited weak α -SMA signals. Furthermore, mouse patellar tendon cells (MPTCs) derived from treadmill running mice were generally larger in culture, proliferated faster, expressed a higher level of α -SMA, and formed more abundant stress fibers compared to MPTCs from control mice. In addition, MPTCs from treadmill running mice generated larger traction forces (169 ± 66.1 Pa) than those from control mice (102 ± 34.2 Pa). Finally, cells from treadmill running mice produced higher levels of total collagen (516.4 ± 92.7 μ g/10,000 cells) than their counterparts (303.9 ± 34.8 μ g/10,000 cells). Thus, mechanical loading via treadmill running increased the presence of myofibroblasts in mouse patellar tendons. As myofibroblasts are activated fibroblasts, their presence in the tendon following treadmill running indicates that they actively repair and remodel tendon tissue under strenuous mechanical loading, leading to known changes in tendon structure. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 27:1373–1378, 2009

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Tendons, which consist of collagen, elastin, proteoglycans, and cells, transmit muscular forces to bones. Tendons alter their structure in response to mechanical loading.¹ For example, exercise enhances both their structural and mechanical properties.² Exercise also increases the collagen level, collagen fibril size, and fibril density.^{2–4} These ultra-structural morphological changes are manifested through improvement in mechanical properties, such as increased ultimate tensile strength of normal tendons following swimming and running.^{5,6} However, the cellular mechanisms responsible for such effects are unclear. One possibility is that, as a result of chronic mechanical loading, a new type of cell becomes present in tendons and is responsible for structural changes. We suspected that the new cells might be myofibroblasts, because mechanical loading of tendon cells in vitro induces differentiation of tendon cells into myofibroblasts.⁷ Myofibroblasts are characterized by α -smooth muscle actin (α -SMA) expression, the formation of α -SMA-containing stress fibers, and the generation of large traction forces that are required for wound closure and extracellular matrix (ECM) remodeling.⁸

The purpose of our study was to test the hypothesis that chronic mechanical loading induces the presence of myofibroblasts in tendons. We applied mechanical loading to mouse patellar tendons in vivo via treadmill running. Following loading, tendon samples were obtained to characterize the cells using immunohistochemistry, cell culture, Western blot, and cell traction

force microscopy. We found that, as a result of mechanical loading, a large number of myofibroblasts were present in tendons.

MATERIALS AND METHODS

Mouse Treadmill Running

C57BL/6J female mice (2.5 months old) were used in a treadmill running study (Exer-6M Open Treadmill; Columbus Instruments). The protocol consisted of 1 week treadmill training, followed by a moderate running exercise at 13 m/min for 50 min/day, 5 days/week, for 3 weeks. Nine mice were used for treadmill running, whereas eight mice served as controls and were allowed to move freely in their cages. The study protocol (#0603480A) was approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Immunohistochemical Staining of Tendon

Sections for α -SMA

Immediately after treadmill running, mice were euthanized using CO₂ asphyxiation. The patellar tendons of two exercised and two control mice were harvested for immunohistochemical analysis. Tendon samples were embedded in OCT 4583 and snap frozen in precooled liquid isopentane. The samples were cut using a cryostat at 13 μ m along the tendon's long axis. Immunostaining protocols based on a previous study⁹ were used to stain the sections for α -SMA, which were then visualized using confocal microscopy.

Tendon Cell Culture and Cell Proliferation

Patellar tendons from seven exercised (14 tendons) and six control mice (12 tendons) were harvested in a sterile manner for cell culture. The tendon sheath and underlying paratenon were removed, and the tendon tissues were minced and placed in a standard tissue culture dish and grown in DMEM (GIBCO-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS), penicillin (50 U/mL), and streptomycin (50 U/mL) at 5% CO₂, 37°C as described in previous

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protocols.¹⁰ Mouse patellar tendon cells (MPTCs) grew out of the tendon explants in about 2 weeks. MPTCs at passages 5 to 8 were used. After these passages, no apparent changes in morphology or doubling time were noted. Cell proliferation was assessed by population doubling time (PDT), which is the total culture time divided by the number of generations. The number of generations was expressed as $\log_2 N_c/N_0$, where N_0 is the population of the cells seeded initially, and N_c is the population at confluence.

Immunostaining α -SMA and Actin Filaments of Cultured Cells

Nonconfluent MPTCs were fixed with 4% paraformaldehyde at room temperature for 15 min. The cells were then washed twice with wash buffer (0.05% Tween 20 in PBS) and permeabilized with 0.1% Triton X-100 for 3 min. After washing, blocking solution (1% BSA in PBS) was applied for 30 min. The primary antibody, mouse α -SMA monoclonal (Sigma-Aldrich, St. Louis, MO), was diluted to 1:500 in the blocking solution and then added. After 1 h incubation with the primary antibody, followed by three washes with washing buffer, the secondary antibody (goat antimouse, FITC-conjugated, 1:500 dilution) was added and incubated for 30 to 60 min before washing. For double labeling, TRITC-conjugated phalloidin was incubated simultaneously with the secondary antibody for 30 to 60 min. Confocal microscopy was used to image stained cells.

Western Blot Analysis

MPTCs at confluence were lysed using mammalian protein extraction reagent (Pierce, Rockford, IL) containing 1.5% protease inhibitors cocktail (Sigma-Aldrich). After centrifugation at 12,000 rpm for 10 min, the protein concentrations of the supernatants were determined with a BCA Protein Assay (Pierce). Equal amounts of total protein (5 μ g for α -SMA, 5 μ g for β -actin, 10 μ g for nonmuscle myosin II) were run on 10% (4–15% for myosin II) SDS-polyacrylamide gels (Bio-Rad, Hercules, CA) at a constant voltage of 90 V. Proteins were blotted to a nitrocellulose membrane using a standard transfer module (Bio-Rad) for 90 min (overnight for myosin II). The membrane was blocked in a 5% dry milk/PBS-Tween 20 solution for 1 h at room temperature and then probed with a mouse monoclonal anti- α -SMA antibody (Sigma-Aldrich) at a dilution of 1:1,500–2,000 in a 1% dry milk/PBS-Tween 20 solution. Incubation with the primary antibody was followed by a peroxidase-conjugated goat antimouse antibody (Jackson ImmunoResearch Lab, Inc., West Grove, PA) at a dilution of 1:2,000 in a 1% dry milk/PBS-Tween 20 solution. The targeted protein bands were detected using an ECL detection kit (Amersham Biosciences, Piscataway, NJ), followed by exposure of the membrane to X-ray film. Membranes were also reprobed for GAPDH to verify equal protein loading in the gels. Finally, α -SMA protein bands were scanned and quantified by image analysis (Scion Imaging 4.0; Scion Corp., Frederick, MD). The density of each band was normalized to that of GAPDH.

Cell Traction Force Microscopy (CTFM)

Two steps were involved in the application of CTFM to determine individual cell traction forces.¹¹ First, polyacrylamide gel disks were prepared with a thickness of 120 μ m and diameter of 10 mm. The Young's modulus of the gel, which contained 5% acrylamide and 0.1% bis, was 3 kPa, and the Poisson's ratio was taken to be 0.48. The gel was impregnated

with 0.2 μ m red fluorescent micro-beads (Molecular Probes, Eugene, OR) and attached to the bottom of a 35 mm glass dish (MatTek, Ashland, MA), which had a 14 mm circular inner glass area and had been consecutively treated with 0.1 M sodium hydroxide, 3-aminopropyltrimethoxysilane, and 0.5% glutaraldehyde. Following polymerization, the gel surface was treated with Sulfo-SANPAH (Pierce) and then coated with 200 μ L of 100 μ g/mL collagen type I solution overnight at 4°C. Subsequently, cells were plated on the collagen-coated gel disks. The cell density was 3,000 cells/disk; the cells were allowed to spread on the gel for 6 h in the same medium as the cell culture treatment. Phase contrast images of individual cells and fluorescent images of the embedded beads were obtained, after which the cells were trypsinized and images of the fluorescent beads in the unstrained gel were taken. Cell traction forces were determined by computation based on a published method.¹² Note that the CTF is defined as the stress (or local force per unit area) imposed on the gel surface by an adherent cell.^{11,12}

Sircol Assay for Total Collagen

The amount of total soluble collagen in cell culture supernatants was quantified using the Sircol collagen assay (Biodye Science, UK). For Sircol assay, confluent MPTCs were used. This assay uses a dye-binding method to quantify total collagen content. The Sircol dye reagent was mixed with cell-media samples for 30 min on an orbital shaker, followed by centrifugation to collect the collagen-dye complex pellet. The pellet was solubilized with an alkali reagent, and the absorbance of the samples was measured at a wavelength of 540 nm using a microplate reader (Spectra MAX 190; Molecular Devices, CA). A calibration standard of acid-soluble type I collagen was used to obtain the standard curve for calculating the amount of collagen present in samples.

Statistical Analyses

A student's *t*-test was used for statistical analyses of α -SMA expression, traction forces, and total collagen production between treadmill running and control mice. The SPSS software package (version 12.0; SPSS, Chicago, IL) was used and a $p < 0.05$ was considered significant.

RESULTS

Immunofluorescence microscopy revealed that numerous cells in tendon sections from treadmill running mice expressed α -SMA. In contrast, only a few cells were present in the tendons of control mice that exhibited a weak α -SMA signal (Fig. 1).

The MPTCs obtained from exercised and control mice were markedly different in morphology. In general, cells from exercised mice exhibited greater surface area (Fig. 2), expressed a higher level of α -SMA, and presented more abundant stress fibers compared to cells from control mice (Fig. 3). These cells grew faster than control cells, with a shorter PDT (44.2 ± 3.9 h) compared to cells from control mice (93 ± 14.4 h; $p < 0.05$) (Fig. 4). In addition, MPTCs from treadmill running mice generated larger traction forces than those from control mice. The cell traction force generated by exercised mice was 102 ± 34.2 Pa compared to 169 ± 66.1 Pa ($p < 0.05$) for control mice (Fig. 5). Finally, MPTCs from exercised mice produced higher levels of total collagen (516.4 ± 92.7 μ g/

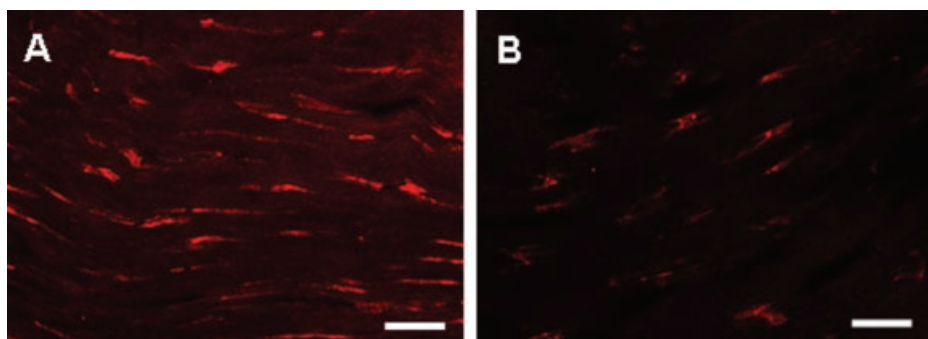


Figure 1. Confocal microscopy of representative tendon sections stained by immunohistochemistry for α -SMA expression of MPTCs from treadmill running mouse (A) and cage control mouse (B). Scale bars: 20 μ m.

10,000 cells) than control mice ($303.9 \pm 34.8 \mu\text{g}/10,000$ cells; $p < 0.05$) (Fig. 6).

DISCUSSION

Mechanical loading has been shown to enhance the structural and mechanical properties of tendons, but the cellular mechanisms responsible for this have not been elucidated. Using a treadmill running model, we showed that numerous cells expressed α -SMA in tendons from mice subjected to running compared to only a few cells that expressed weak α -SMA in control mice. This observation was supported by *in vitro* culture experiments; compared to tendon cells derived from control mice, MPTCs from treadmill running mice were larger, formed prominent stress fibers, and expressed larger amounts of α -SMA, all of which indicated that MPTCs from treadmill running mice are myofibroblasts.^{13,14} To confirm this, we used CTFM to determine cell traction forces, as increased α -SMA expression relates directly to increased traction forces in myofibroblasts.¹⁵ We found that the MPTCs from exercised mice generated greater traction forces than those from control mice. Finally, we showed that the MPTCs of exercised mice produced more collagen than cells from control mice, consistent with previous studies, where running exercise induced collagen synthesis in the tendons of human subjects.^{16–18}

To our knowledge, this study provides the first evidence that myofibroblasts are present in tendons after treadmill running exercise. Myofibroblasts could be responsible for changes in tendon structure, because these cells are considered to be activated fibroblasts that produce abundant collagen¹⁹ and generate large traction forces.¹⁵ Myofibroblasts also play a vital role in the repair

and remodeling of wound tissues.²⁰ A previous study showed that repetitive mechanical loading resulted in micro-tears in tendons.²¹ Therefore, the presence of myofibroblasts suggests that repetitive mechanical loading of tendons may cause micro-injuries, and thus elicits a healing response for repair of injured tendons.

Where the myofibroblasts in this study originated is unclear, but they may have a very heterogeneous origin. In normal conditions, fibroblastic cells exhibit few or no actin-associated cell-cell and cell-matrix contacts and little ECM production.²² After injury, these cells become activated due to the release of cytokines and growth factors such as TGF- β from inflammatory and resident cells.²³ As a result, they migrate into the injured site to produce ECM components for tissue repair. Another possible cell source is circulating fibrocytes, which have been reported to migrate to the site of tissue injury and differentiate into myofibroblasts.²⁴ Fibrocytes play an important role in the genesis of sub-epithelial fibrosis and airway remodeling in asthma.²⁵ In addition, bone marrow stem cells (BMSCs) can circulate and give rise to lung fibroblasts^{26,27} as well as participate in wound healing.²⁸ Nakama et al.²¹ showed that repetitive contraction of the flexor digitorum profundus in rabbits causes tendon micro-tears. Using high resolution ultrasound, Gibbon et al.²⁹ showed an increase in Achilles tendon micro-tears in athletes with symptomatic tendinitis, while the same defects were seen only occasionally in asymptomatic volunteers. Therefore, BMSCs may migrate to injured tendon sites.

In recent years, tissue-specific adult stem cells have been identified in many tissues and organs.³⁰ These stem cells contribute to tissue regeneration after injury by differentiating into specific cell types within the host

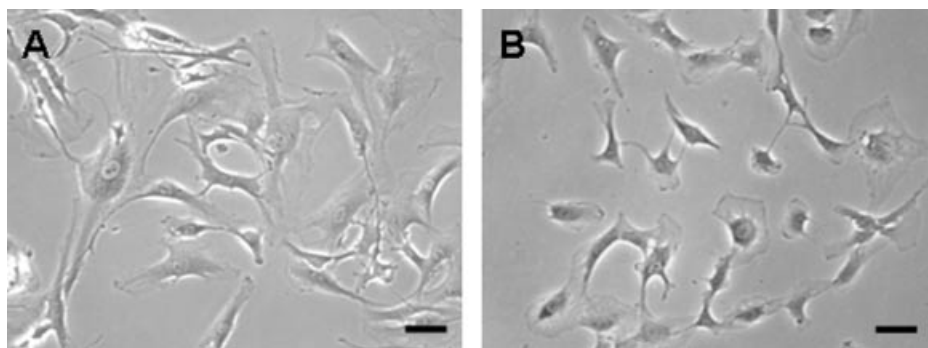


Figure 2. The morphology of MPTCs from treadmill running mouse (A) and cage control mouse (B). MPTCs from treadmill running group show a typical myofibroblast morphology with larger and more spread out cells compared to those from the cage control group. Scale bars: 100 μ m.

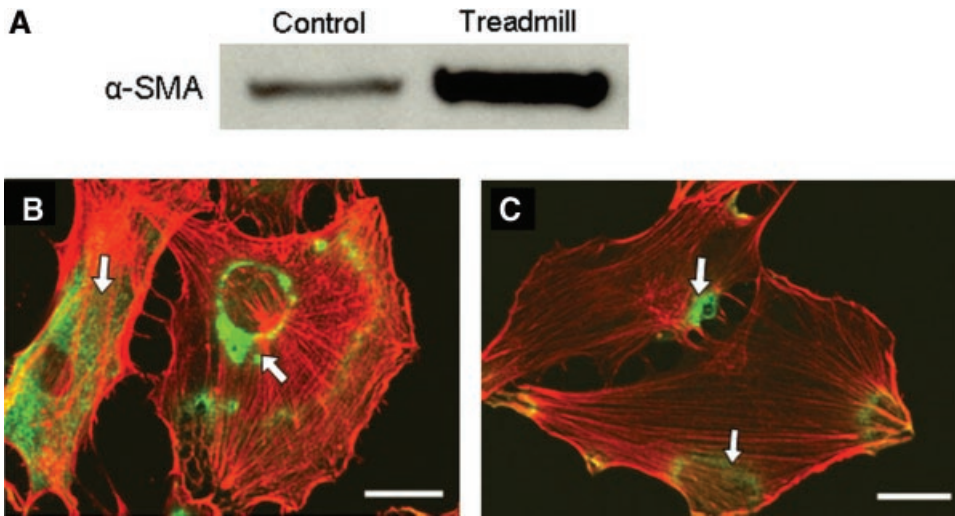


Figure 3. Western blot analysis of α -SMA expression in MPTCs from treadmill running and control mice (A). MPTCs from treadmill running mice had much higher levels of α -SMA expression compared to those cells from cage control mice. Immunocytochemistry for α -SMA (green) and actin filaments (red) of MPTCs from treadmill running mouse (B) and control mouse (C). More α -SMA green signals (arrows) were seen in MPTCs from treadmill running mice than in cells from cage control mice. Scale bars: 20 μ m.

tissue.³¹ In particular, Bi et al.³² isolated tendon stem cells that have multidifferentiation potential. Mechanical loading of stem cells in vitro promotes selective differentiation of stem cells toward specific cell lineages. For example, concurrent application of tensile and rotational loading to human and bovine BMSCs in collagen gels resulted in differentiation to ligament cells.³³ Mechanical loading of bovine BMSCs also enhances osteogenesis.³⁴ Thus, residential tendon stem cells may proliferate and differentiate into myofibroblasts in response to treadmill running. Future studies should investigate whether BMSCs, circulating fibrocytes, or tendon stem cells are active in this response.

While short term, moderate mechanical loading via treadmill running showed an anabolic effect on tendons in our study, excessive mechanical loading is considered to be a major cause of tendinopathy. Using a rat treadmill running model, Archambault et al.³⁵ found that treadmill running for 4 weeks induced a fibrocartilage phenotype in the supraspinatus tendons. In this regard, the anabolic effect on tendons seen in our study, namely increased cellular production of collagen, may reflect the early healing response of tendon cells to the development of tendinosis. In another study, tendinosis occurred in rat

supraspinatus tendons following 12 to 16 weeks of treadmill running.³⁶ The difference between our study and the previous two studies may be attributed to species (mouse vs. rat), type of tendon (patellar vs. supraspinatus tendon), and mechanical loading intensity such as loading duration (3 weeks vs. 4 to 16 weeks). Mechanical loading can be considered to have both beneficial and detrimental effects on tendon matrix. Under moderate loading conditions, it can stimulate collagen synthesis and protect the tendon from degeneration; however, it can cause collagen matrix damage and induce metalloproteinase release when mechanical loading exceeds the physiological limit of the tendon.³⁷ Both these effects appear to be time- and load-dependent.³⁸ Therefore, tendon homeostasis depends on the balance between the intensity of mechanical loading and the ability of the tendon to adapt.

Like any tissue culture experiment, our study is limited by changes in cell phenotype that occurred after extraction of fibroblasts from tendon tissue and subsequent expansion in culture. The subculturing of cells is necessary to provide a sufficient number of cell assays such as Western blot for α -SMA expression. We found that cells from patellar tendons of exercised mice express

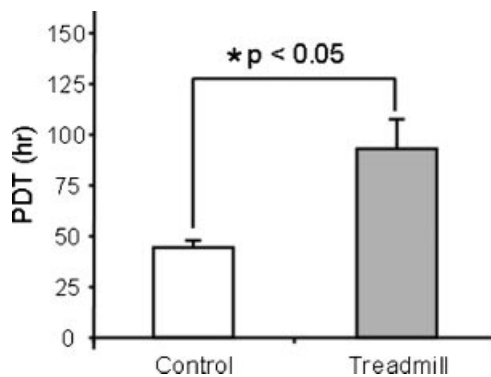


Figure 4. Population doubling time (PDT) of MPTCs. After treadmill running, myofibroblasts from exercised mice proliferated much faster than those cells from cage control mice.

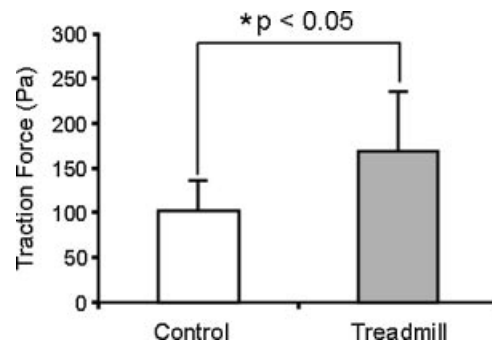


Figure 5. Traction forces of MPTCs from treadmill running and control mice. After treadmill running, MPTCs generated larger traction forces than cells from cage control mice.

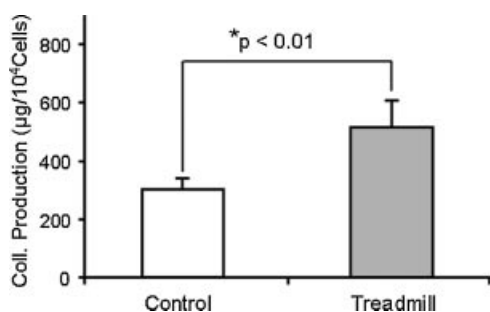


Figure 6. Total collagen production by MPTCs from treadmill running and control mice. Treadmill running significantly increased cellular collagen production compared to cage control mice.

a higher level of α -SMA than those from cage control mice. The results are consistent with the immunostaining results of α -SMA on tendon sections (Fig. 1). Moreover, our previous study showed that after multiple passages, the inherent differences in cell phenotype between healing and normal fibroblasts, at least in terms of α -SMA expression, collagen production, and contraction, were preserved.³⁹ Thus, the limitation of using a cell culture model does not invalidate our current findings.

In summary, we showed the presence of numerous myofibroblasts in the tendons from treadmill running mice and demonstrated that cells from exercised mice grew faster, generated larger traction forces, and produced more collagen than cells from control mice. As myofibroblasts are known to be responsible for repair and remodeling of injured tissues, their presence in tendons after treadmill running suggests that the tendon is in a “healing state”, possibly due to repetitive loading-induced tendon micro-injuries. Further studies should investigate the origin of the myofibroblasts and in particular the possibility of the differentiation of tendon stem cells into myofibroblasts in response to chronic mechanical loading conditions.

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