Production of PGE_2 Increases in Tendons Subjected to Repetitive Mechanical Loading and Induces Differentiation of Tendon Stem Cells into Non-Tenocytes

Jianying Zhang, James H.-C. Wang

MechanoBiology Laboratory, Departments of Orthopaedic Surgery, Bioengineering, and Mechanical Engineering and Materials Science, University of Pittsburgh, E1640 Biomedical Science Tower, 210 Lothrop Street, Pittsburgh, Pennsylvania 15213

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ABSTRACT: Whether tendon inflammation is involved in the development of tendinopathy or degenerative changes of the tendon remains a matter of debate. We explored this question by performing animal and cell culture experiments to determine the production and effects of PGE_2, a major inflammatory mediator in tendons. Mouse tendons were subjected to repetitive mechanical loading via treadmill running, and the effect of PGE_2 on proliferation and differentiation of tendon stem cells (TSCs) was assessed in vitro. Compared to levels in cage control mice, PGE_2 levels in mouse patellar and Achilles tendons were markedly increased in response to a bout of rigorous treadmill running. PGE_2 treatment of TSCs in culture decreased cell proliferation and induced both adipogenesis and osteogenesis of TSCs, as evidenced by accumulation of lipid droplets and calcium deposits, respectively. Effects of PGE_2 on both TSC proliferation and differentiation were apparently PGE_2–dose-dependent. These findings suggest that high levels of PGE_2, which are present in tendons subjected to repetitive mechanical loading conditions in vivo as shown in this study, may result in degenerative changes of the tendon by decreasing proliferation of TSCs in tendons and also inducing differentiation of TSCs into adipocytes and osteocytes. The consequences of this PGE_2 effect on TSCs is the reduction of the pool of tenocytes for repair of tendons injured by mechanical loading, and production of fatty and calcified tissues within the tendon, often seen at the later stages of tendinopathy. © 2009 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 28:198–203, 2010

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Tendons are subjected to large mechanical loading while transmitting muscular forces to bone to enable joint movement. As such, tendons are susceptible to tendinopathy, a collective term of tendon disorder including inflammation and/or degeneration.[1] Using histopathological techniques, previous studies found lipid accumulation and calcification in tendinopathic lesions.[2,3] These findings suggest the presence of cells in tendons with diverse phenotypes that differ from that of the residential tenocytes that express fibroblast phenotype. Indeed, Bi et al. and, more recently, we showed that human, mouse, and rabbit tendons contain tendon stem cells (TSCs) that have the multi-potential to differentiate into tenocytes and cells of non-tenocyte lineages, including adipocytes and osteocytes.[4,5] Nevertheless, the potential role of TSCs in the development of tendinopathy has not been investigated.

Tendinopathy is particularly prevalent in occupational and athletic settings that involve repetitive mechanical loading on tendons; thus, it is generally believed that excessive mechanical loading plays a dominant role. For example, the lifetime cumulative incidence of Achilles tendinopathy in elite endurance athletes is about 10 times higher than that in sedentary persons.[6] To understand the mechanisms for the development of tendinopathy due to mechanical loading, in vitro model studies were performed, showing that cyclic mechanical stretching of tendon fibroblasts (tenocytes) or tendon explants increases production of prostaglandin E_2 (PGE_2).[7–10] PGE_2 is a major mediator of pain and acute inflammation in tendons, and decreases proliferation and collagen production of human tendon fibroblasts.[11] However, whether PGE_2 levels elevate in response to loading in vivo and whether such PGE_2-mediated tendon inflammation is involved in degeneration through its effects on TSCs remain largely unexplored.

We had two related aims: first, to determine whether mechanical loading increases PGE_2 levels in tendons in vivo using a mouse treadmill running model; second, to determine whether PGE_2 alters TSC proliferation and differentiation in vitro using a cell culture model.

MATERIALS AND METHODS

Mouse Treadmill Running

Ten 2.5-month-old C57BL/6J female mice were used. Five mice were used for treadmill running; the remaining five mice were allowed to move freely in their cages. The treadmill protocol included 1 week of training (15 min/day, 5 days/week), followed by running at 13 m/min until the mice were exhausted. Immediately after treadmill running, the mice were euthanized, and patellar and Achilles tendons were harvested. For PGE_2 measurement, tendon samples were prepared by removing the sheaths and paratenon of the dissected tendons. One mouse each in the treadmill running and cage control groups were excluded because of problems in preparation of tendon samples.

Measurement of PGE_2 Levels in Tendons and Bone Marrow

Tendon samples were treated by modification of the procedures described previously.[12] Samples were weighed, minced, placed in 4°C ethyl acetate (100 μg tissue/300 μl ethyl acetate), and homogenized. The samples were centrifuged at 4°C, 2,000*g* for 30 min, and the supernatant was collected. The ethyl acetate was evaporated using a stream of nitrogen gas. The residue was resuspended in enzyme immunosorbent assay (EIA) buffer provided by the kit manufacturer, and stored at

Correspondence to: James H.-C. Wang (T: 412-648-9102; F: 412-648-8548; E-mail: wanghc@pitt.edu)

−80°C until PGE_2 analysis, using a commercially available EIA kit according to the manufacturer's instructions (Cayman Chemical Co, Ann Arbor, MI; Cat. No. 514010). Similar procedures were involved in measuring PGE_2 in femoral bone marrow, which was obtained by flushing the shafts with 4°C ethyl acetate. Values were normalized with respect to tendon or femur weight.

Preparation of Tendon Stem Cell Culture

The procedures for isolating TSCs were as follows.[5] Rabbit patellar and Achilles tendons were dissected from 8- to 10-week-old female New Zealand white rabbits (3.0–4.0 kg). The sheath and surrounding paratenon of the dissected tendons were stripped off, and the tendons were cut into pieces about 1 mm in size. These pieces were digested in collagenase type I (Worthington Biochemical Corp., Lakewood, NJ) and dispase (StemCell Technologies Inc., Vancouver, Canada) solution. The suspensions were centrifuged at 1,500*g* for 15 min, and the supernatant discarded. The remaining cell pellet was resuspended in growth medium DMEM (Lonza, Walkersville, MD) supplemented with 20% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 100 μM 2-mercaptoethanol (Sigma-Aldrich, www.sigmaaldrich.com), 100 U/ml penicillin and 100 μg/ml streptomycin (Atlanta Biologicals). A single-cell suspension was obtained and cultured in flasks at 37°C with 5% CO_2. After 8–10 days in culture, these patellar and Achilles TSCs formed colonies on the culture surface of the flask, while tendon fibroblasts spread. The TSCs were isolated and expanded once to obtain sufficient cells for PGE_2 culture experiments as described below. At confluence, the TSCs retained their cobblestone-like shape (Fig. 1A, B), whereas fibroblasts from the same tendons were highly elongated (Fig. 1C). The number of TSCs among total tendon cells was not determined; a previous study showed that human and mouse tendons contain 3%–4% TSCs.[4]

TSC Proliferation Experiment

TSCs at passage 1 were seeded in 6-well plates at density of 6×10^4/well and cultured in DMEM supplemented with 20% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and three concentrations (1, 10, 100 ng/mL) of PGE_2 (Sigma-Aldrich), chosen based on a previous study.[11] Cells without PGE_2 treatment were used as controls. Cell proliferation was measured at 3 days by counting cell numbers using a hemocytometer.

TSC Differentiation Experiment

TSCs at passage 1 were plated in six-well plates and allowed to reach confluence after initial plating to assure that cell proliferation was minimal during the experiment. The growth medium was DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. PGE_2 with three concentrations (1, 10, 100 ng/ml) was added to cell cultures after cells were grown in cultures overnight. Cell cultures without PGE_2 treatment were used as controls. Every 3 days, culture media were replaced with the addition of fresh PGE_2 to maintain a specific PGE_2concentration. At 21 days, TSCs were stained using Oil Red O and Alizarin Red S assays to examine adipogenesis and osteogenesis of stem cells, respectively.

Oil Red O Assay

After removing the media in cell culture plates, cells were washed with PBS three times each for 5 min and then fixed using 4% paraformaldehyde at room temperature for 40 min. Next, the cells were washed with PBS three times each for 5 min, then with water two times each for 5 min, and incubated with 0.36% Oil Red O solution (Millipore, Cat. # 90358, Billerica, MA). After 50 min, the cells were washed three times with water. The stained cells were examined on an inverted microscope (Nikon eclipse, TE2000-U, Melville, NY), and images taken by a CCD camera and analyzed by SPOT™ imaging software (Diagnostic Instruments, Inc., Sterling Heights, MI).

Alizarin Red S Assay

Cells in culture plates were fixed in ice cold 70% ethanol for 1 h and then rinsed with distilled water two times each for 5 min. Next, the cells were stained with Alizarin Red S (Millipore, Cat. # 2003999) at room temperature for 30 min, followed by examination of stained cells on the inverted microscope. Images of stained cells were taken by the CCD camera and analyzed by the imaging software.

Statistical Analysis

All data are presented as mean ± SD. For each experimental condition, at least three replicates were performed, and the results presented are representative of the triplicates. For PGE_2 measurements, two-tailed Student's *t*-tests were used. Cell proliferation data were analyzed by one-way ANOVA followed by Fisher's PLSD for multiple comparisons. Differences between the two groups were considered significant when p-value was < 0.05.

RESULTS

The five mice in the running group showed different abilities to run on the treadmill. One mouse could run as long as 280 min before exhaustion, whereas another ran

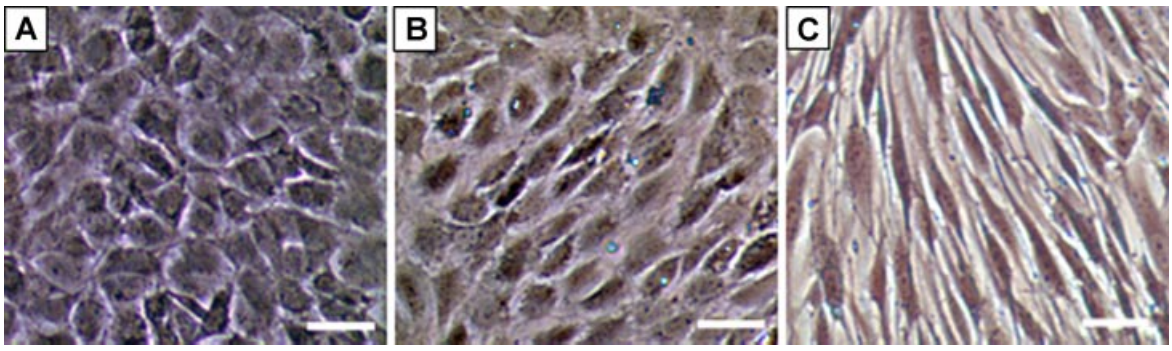

Figure 1. TSCs had a distinctive cobblestone-like shape. (A) TSCs from patellar tendon. (B) TSCs from Achilles tendon. These TSCs were used in culture experiments for determining the effect of PGE_2 treatment on cell proliferation and differentiation. (C) Tenocytes in culture. These cells exhibit a highly elongated shape in confluent conditions. Scale bars: 50 μm.

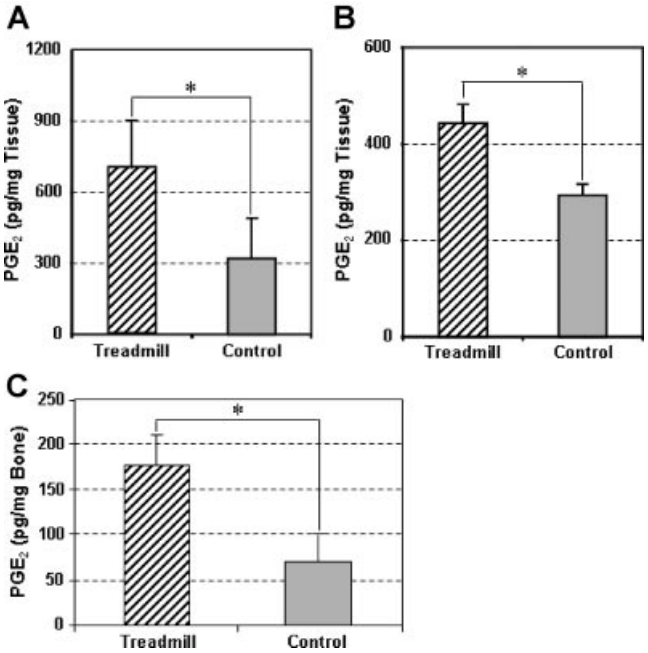

Figure 2. Repetitive mechanical loading increased PGE_2 production in patellar and Achilles tendons. (A) Patellar tendon ($^*p = 0.029$). (B) Achilles tendon ($^*p = 0.001$). (C) PGE_2 levels in mouse bone marrow were also increased in response to treadmill running ($^*p = 0.017$).

only 150 min. The mean running time was 212 ± 50 min. In response to such a bout of treadmill running, PGE_2 levels in the patellar and Achilles tendons increased by 119% (Fig. 2A) and 51% (Fig. 2B), respectively, compared to their respective controls. In addition, PGE_2 levels in mouse bone marrow also increased significantly in response to running (Fig. 2C). PGE_2 levels in marrow serve as positive controls for those in tendons.

Cell proliferation decreased significantly compared to non-treated control cells. The extent of the decrease in TSC proliferation was PGE_2–dose-dependent (Fig. 3). Moreover, PGE_2 treatment induced differentiation of TSCs into adipocytes and osteocytes, as lipid droplets (Fig. 4A) and calcium deposits (Fig. 5A) were

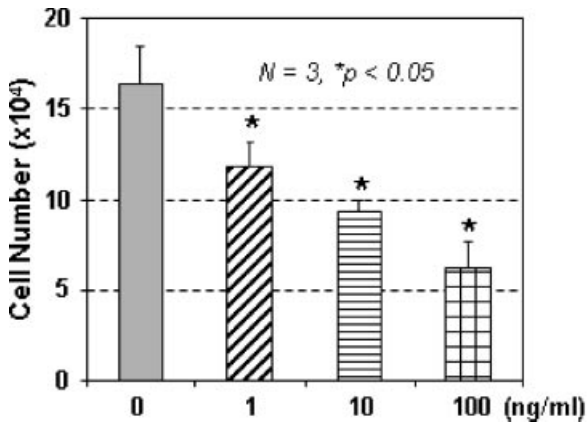

Figure 3. PGE_2 treatment of TSCs decreased TSC proliferation in an apparent dose-dependent manner. All comparisons were made with respective control cells (i.e., no PGE_2 treatment) ($^*p < 0.05$, compared to control cells).

accumulated on cell surfaces. The PGE_2 effect on adipogenesis and osteogenesis of TSCs also appeared to be PGE_2–dose-dependent; the higher the dosage, the more extensive the adipogenesis (Fig. 4B) or osteogenesis (Fig. 5B) in terms of staining area.

DISCUSSION

To explore the relationship between tendon inflammation and tendon degeneration, we measured PGE_2 production in mouse tendons using a treadmill running model and determined PGE_2 effect on TSC proliferation and differentiation in vitro. After a bout of rigorous treadmill running, higher levels of PGE_2 were present in mouse tendons compared to cage control mice. Moreover, PGE_2 decreased TSC proliferation and induced adipogenic and osteogenic differentiation of TSCs in an apparent dose-dependent fashion. These results show that high levels of PGE_2 are produced by tendons in response to repetitive mechanical loading in vivo and also suggest that the presence of high levels of PGE_2 has a detrimental effect on tendons by decreasing TSC proliferation in the tendons and inducing these stem cells to differentiate into non-tenocyte lineages. One consequence of these PGE_2 effects could be depletion of the pool of tenocytes, as fewer TSCs are produced to differentiate into tenocytes, and hence fewer tenocytes are available for repair of tendon tissues injured as a result of intensive loading. Another consequence could be formation of fatty and bony tissues inside the tendons by adipocytes and osteocytes, respectively, which is often seen in tendinopathic tendons at the later stages.[2,3]

To the best of our knowledge, this was the first study that determined PGE_2 levels in animal tendons in response to repetitive mechanical loading via treadmill running. The results, however, are supported by the mechanical loading increase in PGE_2 levels in mouse bone marrow (Fig. 2C), which is consistent with the previous finding that mechanical loading of bone increases PGE_2 production.[13] Our results of increased PGE_2 levels in tendons after treadmill running are also consistent with those of previous studies using in vitro and in vivo models. For example, PGE_2 production markedly increases when human tendon fibroblasts and avian tendon explants are subjected to cyclic mechanical loading.[7,8,10] Exercise in the form of intermittent static plantar flexion of the ankle also increases PGE_2 levels in the peritendinous space of human Achilles tendon.[14] Taken together, the increased PGE_2 levels in mouse tendons obtained in our study were likely due to PGE_2 production by the tendons in response to repetitive loading.

However, the systemic stress on the mice from strenuous running might have contributed to elevated PGE_2 levels in tendons. We tried to minimize the systemic stress effect by allowing mice to undergo 1 week of training, to run at the same time every day, and to run at a moderate speed (13 m/min). These measures made treadmill running as smooth as possible. Nevertheless,

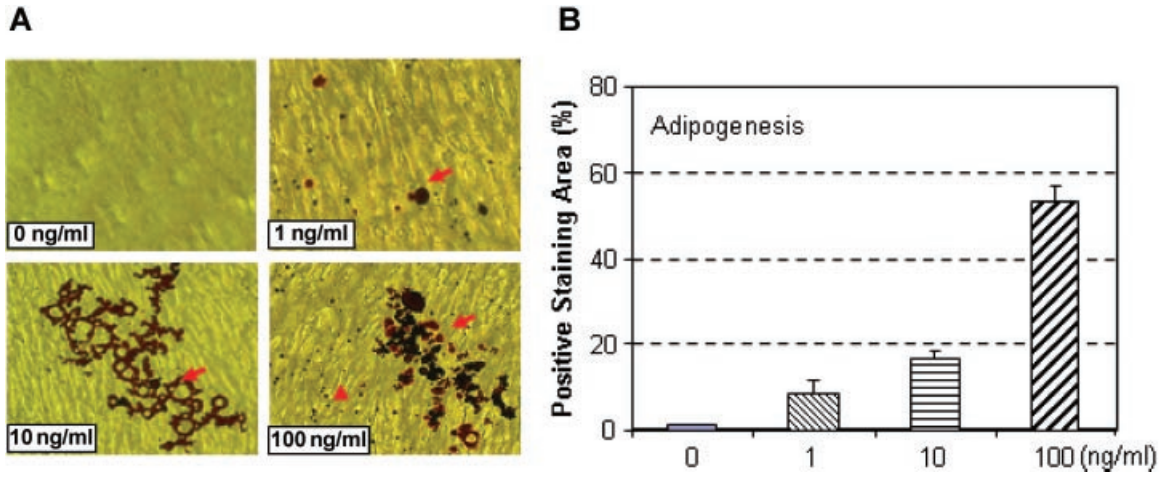

Figure 4. (A) PGE_2 induced adipogenic differentiation of TSCs. Cells were cultured in DMEM + 10% FBS with three concentrations of PGE_2 for 21 days. Arrows point to accumulated lipids, and triangle indicates the area where a large number of fat droplets are present (Oil Red O staining; original magnification: 10×). (B) Adipogenesis increased with increased PGE_2 concentration in culture. Imaging software was used to measure positive staining areas, and the percentage of the staining area with respect to each image area (four total) was calculated.

the running duration was relatively long (~4 h on average), so the running was intensive and may have caused "stress" on the mice, affecting PGE_2 production in tendons. It remains to be seen in future studies whether a less intensive running protocol also elevates PGE_2 production in mouse tendons; an exercise study on human subjects suggests this may be the case.[14]

In cell culture experiments, PGE_2 was present in TSC culture for 3 weeks before the phenotypes of adipocytes and osteocytes were detected, consistent with the development of tendinopathy from repetitive exposure to high levels of PGE_2, likely produced by repetitive mechanical loading over a long period of time. Thus, one bout of intensive running is unlikely to cause a tendon problem as the elevated PGE_2 level may subside after rest. However, repetitive intensive running over time could induce the development of tendinopathy, as this will expose TSCs to continued high levels of PGE_2, causing reduced cell proliferation and abnormal cell differentiation, as shown in this study, and eventually resulting in tendinopathy.

Previous studies did not focus on stem cells, but they included examinations into the diverse biological effects of PGE_2 on many types of adult cells and include cell proliferative,[15,16] inflammatory, and immune responses.[17,18] Elevated PGE_2 levels have been implicated in a number of inflammatory diseases, such as neurodegenerative disorders,[19,20] and in the formation of lesions in gingival tissue.[21] In vitro, PGE_2 decreases proliferation and collagen synthesis of fibroblasts.[11,22–24] PGE_2 also induces fibroblasts to synthesize matrix metalloproteinases (MMPs), which cause connective

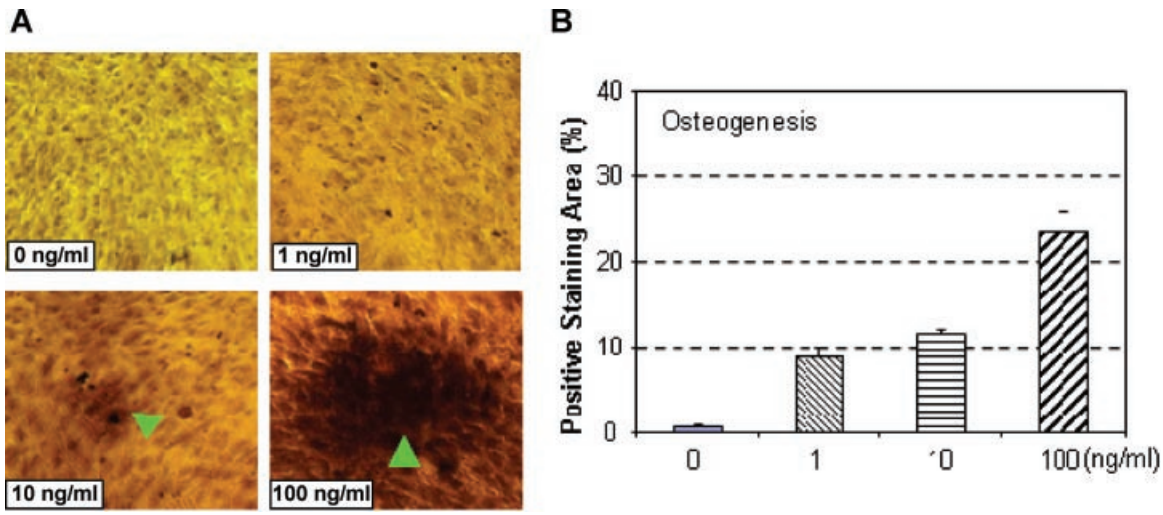

Figure 5. (A) PGE_2 induced osteogenic differentiation of TSCs. The cells were cultured in DMEM + 10% FBS with three concentrations of PGE_2 for 21 days. The darker the staining color, the more extensive the accumulation of calcium deposits (triangles; Alizarin Red S staining; original magnification: 10×). (B) Image analysis results showed an increase in osteogenesis with respect to increased PGE_2 concentration. The positive staining areas were measured using imaging software, and the percentage of the positive staining area with respect to each image area (four total) was then calculated.

tissue degradation, the hallmark of tendinopathy at later stages.[25,26] Finally, in a previous study, injection of PGE_2 into rabbit tendons led to the presence of fat cells in localized sites.[27] This result lends support to our finding that PGE_2 induced differentiation of TSCs into adipocytes and osteocytes. Thus, high PGE_2 levels present in tendons due to repetitive mechanical loading conditions in vivo may play a major role in the development of tendinopathy, which may be at least mediated by non-tenocyte differentiation of TSCs in tendons.

Although the molecular mechanisms by which PGE_2 decreases TSC proliferation in this study are unclear, they may involve PGE_2 cell surface receptors.[28] Four subtypes of receptors, designated EP1–4, have been identified.[29,30] They are encoded by different genes, and each has unique a signal transduction mechanism as a result of coupling to different G proteins.[28] The cellular mechanisms for PGE_2 induction of adipogenic and osteogenic differentiation of TSCs likely involve PGE_2 surface receptors. Moreover, there may be two subpopulations of stem cells within the TSCs used in this study, one inclined toward adipocyte differentiation and the other osteocyte differentiation. Future studies should examine the role of PGE_2 surface receptors in the PGE_2 effect on TSC proliferation, and investigate the composition of subpopulations of TSCs.

A few limitations are in place for proper interpretation of our results. First, the local PGE_2 concentrations secreted by local tendon cells and affecting local TSCs were unknown and likely underestimated, as our PGE_2 was done and "averaged" on whole tendons. Also, because mouse tendons are small and yield a limited number of mouse TSCs, we used rabbit TSCs, which are more abundant, in cell culture experiments. Because of these two reasons, we chose three PGE_2 concentrations known to exert catabolic effects on tendon fibroblasts with decreased cell proliferation and collagen production.[11] Future studies should include a way to measure local PGE_2 concentrations in tendons, and use these concentrations to determine the effect on TSCs from the same species. This should yield more accurate information regarding the effects of PGE_2 on TSCs, including proliferation and differentiation.

Second, our cell culture model is limited in that it did not include tenocytes, the dominant cell type in tendons, and mechanical loading was not included. Both tenocytes and mechanical loading should regulate TSC mechanobiology. An improved cell culture model that considers these two factors would provide new information on the effect of PGE_2 on the interactions between TSCs and tenocytes under a more physiologically relevant experimental condition.

Third, tendons likely produce many factors other than PGE_2 in response to mechanical loading. For example, the tissues may also produce leukotriene B_4 (LTB_4), which counterbalances the catabolic effects of PGE_2 on tendon fibroblasts.[31] The potential effects of LTB_4 on TSCs should be investigated in future studies. Besides mechanical loading and loading-induced PGE_2, other extrinsic and intrinsic factors, such as traumatic events, IL-1β, and neuropeptides, can contribute to the development of tendinopathy.[25,32] The involvement of multiple factors may explain why, in some cases, an increased presence of cells was found in degenerative tendons.[32] Finally, in light of the finding that TSCs are present in tendons and can differentiate into multiple cell types, rigorous gene and protein analyses are required to determine the cell types present in tendons in response to repetitive mechanical loading.[33]

In summary, this study showed for the first time that after a bout of rigorous treadmill running, mouse tendons produced higher levels of PGE_2 than tendons of cage control mice, and PGE_2 decreased TSC proliferation and induced adipogenic and osteogenic differentiation of TSCs in an apparent dose-dependent fashion. These data support the hypothesis that high levels of PGE_2 produced by tendons in response to intensive, repetitive mechanical loading in vivo may lead to the development of tendinopathy by two parallel cellular mechanisms: decreasing the number of TSCs, and inducing differentiation of TSCs into adipocytes and osteocytes. This decrease in TSC number, and the induction of adipogenesis and osteogenesis by PGE_2, may deplete the number of TSCs available for tendon repair, and lead to the lipid accumulation and calcification often seen in lesions of tendinopathic tendons. Further research is required to investigate this possibility and the molecular mechanisms responsible for PGE_2-induced effects on TSC proliferation and differentiation.

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