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Rabbit tendon cells produce MMP-3 in response to fluid flow without significant calcium transients

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Abstract

Forces applied to tendon during movement cause cellular deformation, as well as fluid movement. The goal of this study was to test the hypothesis that rabbit tendon fibroblasts detect and respond to fluid-induced shear stress. Cells were isolated from the paratenon of the rabbit Achilles tendon and then subjected to fluid flow at 1 dyn/cm^2 for 6 h in a specially designed multi-slide flow device. The application of fluid flow led to an increased expression of the collagenase-1 (MMP-1), stromelysin-1 (MMP-3), cyclooxygenase II (COX-2) and interleukin-1β (IL-1β) genes. The release of proMMP-3 into the medium exhibited a dose-response with the level of fluid shear stress. However, not all cells aligned in the direction of flow. In other experiments, the same cells were incubated with the calcium-reactive dye FURA-2 AM, then subjected to laminar fluid flow in a parallel plate flow chamber. The cells did not significantly increase intracellular calcium concentration when exposed to fluid shear stress levels of up to 25 dyn/cm^2. These results show that gene expression in rabbit tendon cells is sensitive to fluid flow, but that signal transduction is not dependent on intracellular calcium transients. The upregulation of the MMP-1, MMP-3 and COX-2 genes shows that fluid flow could be an important mechanical stimulus for tendon remodelling or injury.

Keywords: Calcium; Tendon cells; Fluid-induced shear stress; Cell mechanics; Matrix metalloproteinases

1. Introduction

Cells regulate the biosynthetic activity of musculoskeletal tissues (Frank and Hart, 1990). It is generally accepted that this activity is, at least in part, regulated by the mechanical stress–strain state of the cell, and that the cellular stress–strain state depends directly on mechanical loading. For example, when a muscle contracts, the corresponding tendinous tissue may experience a variety of loading conditions: tension in the free running tendon, compression where the tendon contacts a bony prominence, or shear stress when individual fascicles move past each other or the tendon moves within its sheath (Benjamin and Ralphs, 1997). Therefore, cells on the tendon surface (epitenon and paratenon) are likely subjected to fluid-induced shear stress as interstitial fluid is circulated through tendon matrix past cells. We believe that tendon cells are subjected to fluid-induced shear stress as a consequence of reciprocal displacement, surface contact with extratendinous structures and interstitial fluid movement. Unfortunately, the levels of fluid-induced shear stress in tendons have not been measured or modelled, but numerous investigators have reported that friction occurs between tendons and sheaths (Goldstein et al., 1987; Uchiyama et al., 1995; Smutz et al., 1994).

One of the most rapid cellular responses to a mechanical stimulus is an increase in intracellular calcium (Banes et al., 1995). Elevated calcium levels

Abbreviations: AP-1: activator protein; COX-2: Cyclooxygenase II; cDNA: Complementary deoxyribonucleic acid; DMEM: Dubelcco's Modified Eagle's Medium with high glucose; DNase: deoxyribonuclease; EBSS: Earle's balanced salt solution; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HEPES: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; IL-1β: interleukin-1β; MMP-1: matrix metalloproteinases-1; Collagenase-1; MMP-3: matrix metalloproteinases-3; stromelysin-1; PGE_2: prostaglandin E_2; RNA: ribonucleic acid

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initiate the transcription of many immediate early genes, which in turn affect the expression of late response genes (van Haasteren et al., 1999). Cells from bone, ligament and cartilage mobilize intracellular calcium in response to fluid flow (Hung et al., 1995, 1997; Yellowley et al., 1997). Osteoblasts, chondrocytes and endothelial cells exposed to fluid flow also alter gene expression over time (Malek and Izumo, 1995; Pavalko et al., 1998; Hung et al., 2000), but this response has yet to be evaluated with tendon cells.

We have previously used the rabbit Achilles tendon as a model to study how tendons respond to mechanical loading in vivo, and what factors are involved in the development of overuse injuries (Archambault et al., 2001). Since the relative displacement of a tendon in a sheath has been proposed to be a mechanism for tendon injury (Moore et al., 1991), we wanted to evaluate if tendon cells responded to the fluid flow that might occur as a result of this displacement. We hypothesized that rabbit tendon cells would respond to fluid-induced shear stress by increasing intracellular calcium, realigning in the direction of flow and altering gene expression. Particularly, we were interested in the expression of IL-1β, COX-2, MMP-1, and MMP-3, factors that might be involved in tendon injury and matrix degeneration.

2. Materials and methods

Cell populations were isolated from tendon according to the method described by Banes and co-workers (Banes et al., 1988). Cells from the Achilles tendon of male New Zealand White rabbits (age 5–7 months) were used in this study. The tendons were removed aseptically from rabbits sacrificed in unrelated experiments. The paratenon was separated from the Achilles tendon, incubated at 37°C in 0.5% collagenase for 5 min, then in 0.25% trypsin for 15 min. These enzymatic treatments released fibroblasts from the paratenon matrix. Cells were plated in DMEM supplemented with 20 mM HEPES, pH 7.2, 100 U/ml penicillin G, 100 μg/ml streptomycin sulphate, and 10% fetal bovine serum (all cell culture products from Gibco BRL, Rockeville MD). Primary cells were grown to confluence, then passaged at a ratio of 1:3. These rabbit paratenon cells resemble the well-described avian tendon surface cells in size ($\approx 200\,\mu m \times 35\,\mu m$) and doubling time ($\approx 23$ h) (Banes et al., 1988).

To study changes in intracellular calcium, passage 2 cells were cultured at 10,000 cells/cm^2 in a 3 mm diameter circle in the central region of $75 \times 24 \times 0.18$ mm type I collagen peptide bonded glass coverslips (CultureSlips^{TM}, Flexcell International Corp., McKeesport, PA). Cells were plated in distinct spots rather than as a monolayer over the entire coverslip to minimize the effect of molecules that can be secreted from cells upstream to those being observed. Cells were growth arrested by replacing half the culture medium on days 3 and 5 with DMEM without serum. Experiments were performed with the cells on days 6 or 7 after plating. Non-dividing, quiescent cells in the G_0 phase of the cell cycle were used so that calcium fluxes associated with cell division would not be a confounding factor.

A parallel plate flow chamber (FlexFlow^{TM}, Flexcell International Corp.) interfaced with a peristaltic pump (MasterFlex® L/S, Cole-Parmer Instrument Company, Vernon Hills, IL) was used to apply fluid flow to the cells. The flow rate was varied from 0 to 163 ml/min to achieve fluid-induced shear stresses of 1–25 dyn/cm^2 according to the equation: $\tau = 6\mu Q/bh^2$ where τ is shear stress in dyn/cm^2, μ is viscosity in dyn/cm^2, Q is fluid flow rate in cm^3/s, b is the flow channel width in cm and h is the flow channel height in cm (White, 1986). The flow perfusate was room temperature, serum-free EBSS or DMEM supplemented with 20 mM HEPES. The calcium chloride concentration in both these perfusates was 200 mg/l. Experiments were repeated on cells from the four different rabbits.

Rabbit Achilles tendon cells were also tested for the ability to respond to a membrane indentation with a micropipet. This poke model was used as a positive control for the ability to increase intracellular calcium in response to a mechanical stimulus (Sanderson et al., 1990). Cells were cultured in spots on coverslips as above; a 1 μm wide glass micropipet was advanced 2 μm into a single cell then retracted. Experiments were repeated on cells from two different rabbits.

The fluorescent dye FURA-2 AM (F-1221, Molecular Probes, Eugene, OR) was used to monitor intracellular calcium concentration ($[Ca^{2+}]_{ic}$). Cells were incubated with 5 μM FURA-2 for 2 h at room temperature, then washed. The cells were imaged with a fluorescence microscope (Olympus BH-2, Tokyo, Japan) equipped with a long working distance 20X ultraviolet objective. All cells within a field of view (typically 40–60 cells) were outlined manually using an image analysis software program (Image-1, Universal Imaging, West Chester, PA). Background intensities were subtracted from images taken every 1.5 s at 380 nm (free calcium) and 340 nm (bound calcium) excitation wavelengths. The ratio of these fluorometric images was calculated. The intensity ratio was converted to a calcium concentration by comparison to values from a standard calcium curve (C-3009, Molecular Probes).

For all fluid flow trials, intracellular calcium concentration was recorded for 30 s prior to flow initiation, during 60–90 s application of the flow stimulus, and for 30 s immediately following the flow stimulus. The mean $[Ca^{2+}]_{ic}$ level during the no-flow baseline and the flow period were calculated. Since there is no accepted definition of what constitutes a biologically or statistically significant increase in $[Ca^{2+}]_{ic}$, we chose to define a

responding cell as one where its mean $[Ca^{2+}]_{ic}$ during the flow period was more than three standard deviations above its mean baseline level. Since three standard deviations about a mean represent a probability of 99.7%, we are confident that any level of $[Ca^{2+}]_{ic}$ outside these boundaries represents a statistically significant change in $[Ca^{2+}]_{ic}$ over the cell's baseline level since it has a probability of only 0.3% of occurring by chance; in effect, this is a p value of 0.003. The percentage of cells responding to this degree was calculated for each fluid flow trial. The peak value of $[Ca^{2+}]_{ic}$ during flow was determined for each cell. The absolute increase in $[Ca^{2+}]_{ic}$ was defined as the difference between the peak and baseline values. For trials where the cells were poked, the data were analyzed in a similar manner.

To study gene expression, cell alignment and protein secretion in response to fluid flow, cells at passage 2 were plated in a monolayer at a density of 10,000 cells/cm^2 on $75 \times 25 \times 1$ mm type I collagen peptide bonded glass coverslips (CultureSlips^{TM}, Flexcell International Corp.) and cultured in DMEM with 2% serum, antibiotics and HEPES. After the 5th day of culture, and 24 h before the experiment, the medium was changed to serum-free DMEM. Six coverslips were placed in a multi-slide flow device (Fig. 1; Streamer^{TM} Flexcell International Corp.) designed to allow long-term application of fluid flow in a sterile environment. The flow perfusate was serum-free DMEM with antibiotics.

In the first experiment, cells on six coverslips were subjected to fluid flow for 6 h at a flow rate that corresponded to a shear stress of 1 dyn/cm^2. The six control coverslips were placed in the flow chamber without the application of flow for 2 min, then removed and placed in an incubator for the duration of the experiment. Immediately after the flow period, cells from the two control slides and two experimental slides were disrupted in a lysis buffer containing guanidinium thiocyanate. The remaining slides were incubated at 37°C for 9 h in 15 ml of serum-free DMEM. After the incubation period, the cells were lysed and the culture supernatant fluid frozen.

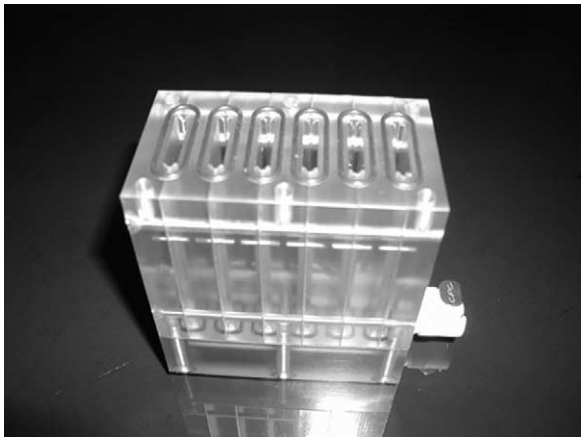


Fig. 1. Picture of the multi-slide flow device which holds six slides. Fluid enters at the bottom, is circulated past the slide in each chamber, then exits at the top. The top lid of the chamber is not shown in the picture.

Total cellular RNA was isolated from all the lysates, treated with DNase and quantified by measuring the absorbency at 260 nm (all products used for RNA isolation were from QIAGEN, Germany). One microgram of RNA was reverse transcribed and subjected to 25 or 30 cycles of polymerase chain reaction (linear range of amplification) according to the protocol described by the manufacturer (GeneAmp RNA PCR kit, Perkin-Elmer Biosystems, Foster City, CA). The following primer sequences were used to amplify cDNAs of interest (Hart et al., 1998):

COX-2	Forward	5′ CTC TAA AGA CGT GGT GGA GAA AC 3′
	Reverse	5′ AGA TCA TCT CCA CCT GCG TGT CCT 3′
MMP-1	Forward	5′ GGT GAT GTT CAG TTA GCT CAG G 3′
	Reverse	5′ CTG TGA ATG TCC TTG GGG TA 3′
MMP-3	Forward	5′ GCC AAG AGA TGC TGT TGA TG 3′
	Reverse	5′ AGG TCT GTG AAG GCG TTG TA 3′
IL-1β	Forward	5′ TAC AAC AAG AGC TTC CGG CA 3′
	Reverse	5′ GGC CAC AGG TAT CTT GTC GT 3′
GAPDH	Forward	5′ TCA CCA TCT TCC AGG AGC GA 3′
	Reverse	5′ CAC AAT GCC GAA GTG GTC GT 3′

Products were separated in 1.8% agarose gels, stained with ethidium bromide, then photographed.

The PGE_2 content of the supernatant fluid was measured with a commercially available enzyme-immunoassay kit (RPN222, Amersham Pharmacia Biotech, UK). The remaining supernatant fluid was concentrated with centrifugal filters (Centriplus YM-30, Millipore, Bedford, MA); MMP-3 proenzyme in the concentrate was measured with a rabbit specific ELISA (RPN2615, Amersham Pharmacia Biotech). The median values of PGE_2 and MMP-3 in the supernatant fluid of the control and experimental cells ($n = 4$ coverslips each) were calculated, and a Mann-Whitney U procedure used to test the hypothesis that the experimental cells would have greater levels of these two molecules in the culture supernatant fluid than the control cells.

In a second experiment, cells were exposed to fluid flow corresponding to a shear stress of 0, 1, 10 or 25 dyn/cm^2 (six coverslips each) for 3 h. Since the magnitude of fluid-induced shear stress in tendon in vivo is not known, we chose levels that corresponded to those used for other cell types in vitro. After the 3 h flow period, two coverslips from the control group and two from the experimental group were fixed in 10% formalin at 4°C overnight. To identify the actin cytoskeleton, coverslips were stained with 6.6 μM rhodamine phalloidin (R-415, Molecular Probes) for 20 min at room temperature according to instructions from the supplier. Cells were imaged with a fluorescence microscope (Axiovert S100 TV, Carl Zeiss Inc., Thornwood, NY) and photographed digitally. Three photographs were taken at random locations on each coverslip.

The four remaining control and experimental coverslips were incubated in 15 ml serum-free DMEM for the next 9 h. The medium was then concentrated and assayed with the MMP-3 ELISA as above. Median values were calculated for each condition, then analyzed with a Kruskal–Wallis test. A p value below 0.05 was considered significant for all statistical tests.

3. Results

The 6 h flow protocol at 1 dyn/cm^2 resulted in marked changes in gene expression (Fig. 2). Immediately post-flow, COX-2 was induced in experimental cells. After the 9 h incubation, COX-2 expression was still elevated, and MMP-1, MMP-3 and IL-1β were also expressed in the experimental cells; note that these genes were barely, or not detectable in control cells (C0 and C9 in Fig. 2). To substantiate the changes in gene expression, we also measured the levels of MMP-3 protein released into the culture supernatant fluid. Cells subjected to fluid flow released significantly more MMP-3 than control cells ($p = 0.029$). We also measured PGE_2 in the medium as its synthesis is the downstream consequence of elevated COX-2 expression. Experimental cells did not release significantly more PGE_2 than control cells ($p = 0.0886$). In the dose response experiment, the release of MMP-3 significantly increased with the level of shear stress ($p = 0.038$, Table 1). We observed some degree of cell alignment in the direction of flow, however, this was not apparent in all cells, nor in all areas of the coverslips (Fig. 3).

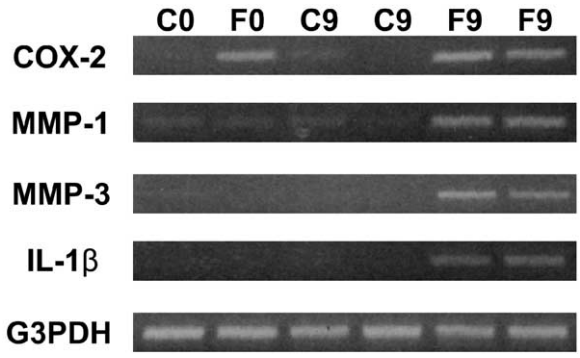


Fig. 2. Gene expression evaluated by RT-PCR in control (C) and experimental (F) samples immediately after 6 h of flow at 1 dyn/cm^2 (C0 and F0) and after 9 h of incubation (C9 and F9, RNA samples from different coverslips). Immediately at the end of the flow period, COX-2 was upregulated. COX-2, MMP-1, MMP-3 and IL-1β were upregulated at 9 h post-flow. Equal GAPDH levels in each sample show that sample loading of total RNA was equal.

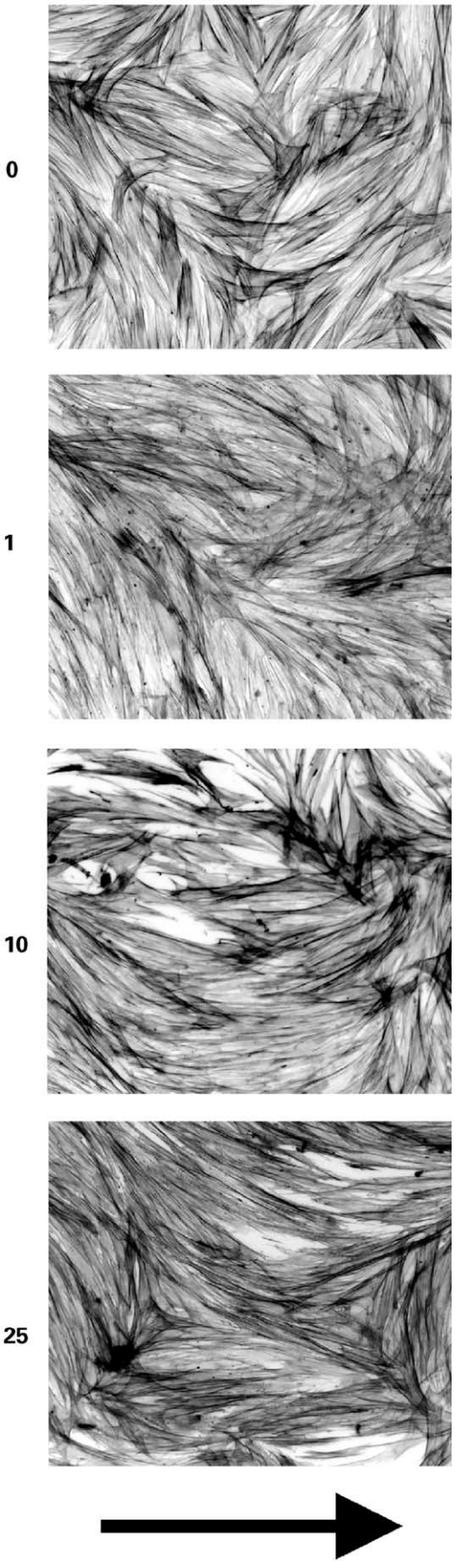
None of the cells significantly increased intracellular calcium levels in response to any level of fluid shear stress up to 25 dyn/cm^2. The average increase in $[Ca^{2+}]_{ic}$ for a typical trial at 10 dyn/cm^2 was 13.7 nM (Fig. 4); with a baseline of 65.8 nM, this represents a 20% increase. This lack of increase in intracellular calcium was observed in paratenon cells from all rabbits and was independent of passage level and phase of cell cycle (data not shown). Moreover, cells from two other tendons, the patellar and flexor digitorum profundus, did not mobilize intracellular calcium in response to shear stress (data not shown). When the membrane of one cell was deformed with a micropipet, the poked cell and all of its neighbouring cells had an immediate and significant increase in intracellular calcium concentration (Fig. 5).

4. Discussion

Shear stress is one type of mechanical stimulus to which tendon cells are likely subjected in vivo. The

Table 1
Quartile statistics for proMMP-3 released into the supernatant fluid (ng/ml) in the 9 h following a fluid-flow experiment at the indicated levels ($n = 4$ in each group). There was a significant difference between groups ($p = 0.038$)

Shear stress (dyn/cm^2)	25th percentile	50th percentile (median)	75th percentile
0	71.9	101.4	168.4
1	69.8	134.2	174.0
10	117.2	128.0	218.6
25	256.4	861.0	1849.5



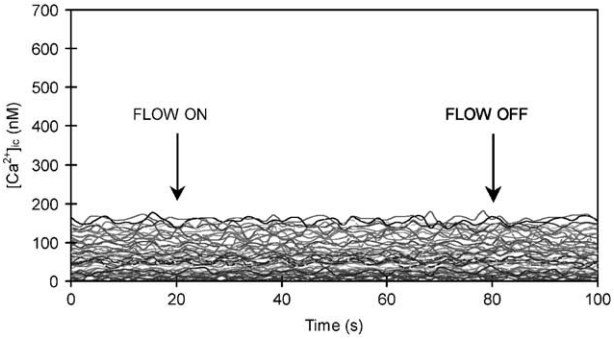


Fig. 4. Real-time intracellular calcium levels in a representative trial where EBSS was perfused at 10 dyn/cm^2. Each line represents an individual cell. There were no significant transient increases in intracellular calcium in response to the fluid-flow stimulus.

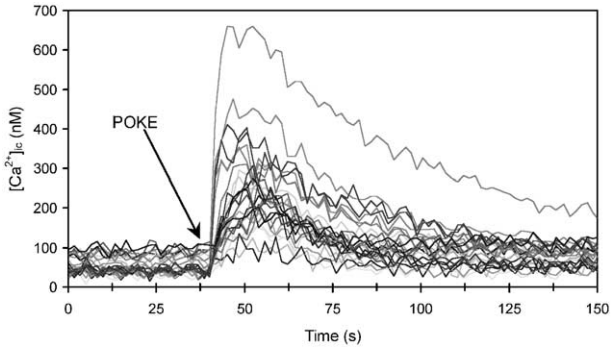


Fig. 5. Real-time calcium levels in a representative trial where the cell membrane of one cell was indented with a micropipet. The target cell and its neighbours increased intracellular calcium immediately after the poke, indicating that tendon cells can respond to a mechanical deformation of their membrane. Each line represents one cell.

objective of this research was to evaluate if rabbit tendon cells respond to fluid flow. This study is the first to report that tendon cells can alter gene expression in response to fluid flow. To our knowledge, this is also the first report of the sensitivity of the MMP-1 and MMP-3 genes to fluid flow. This induction is not a complete surprise, however, as AP-1 binding has been shown to increase when fluid flow is applied (Lan et al., 1994) and AP-1 is necessary for the expression of MMP-1 in response to a stretching stimulus (Park et al., 1999). It has been recently reported that MMP-9 is also responsive to a fluid shear stress of 16 dyn/cm^2 (Jin et al., 2000). In our experiments, tendon cells increased MMP-3 expression and production at levels of shear

Fig. 3. Micrographs of cells stained with rhodamine phalloidin to visualize the actin cytoskeleton. Cells were either not subjected to flow (0) or subjected to fluid flow at different levels of shear stress (1, 10, 25 dyn/cm^2) for 3 h. The arrow indicates the direction of fluid flow. Some of the cells aligned with the direction of flow.

stress lower than those reported by Jin and co-workers (2000).

It is well known from the literature that endothelial and bone cells release prostaglandins in response to fluid flow (Reich and Frangos, 1991; McAllister et al., 2000; Doroudi et al., 2000). We were not able to detect any PGE_2 in the flow perfusate, probably because the volume of perfusate in our multi-slide system was so large (500 ml) that the PGE_2 concentration was diluted to levels below the detection threshold of our assay (data not shown). We were surprised however that we did not measure greater levels of PGE_2 after the post-flow incubation period, even though the COX-2 gene was clearly upregulated as a result of flow. Several explanations exist: (1) PGE_2 was released into the culture supernatant fluid early on in the culture period, then degraded; (2) PGE_2 was released from intracellular stores during the flow period, and the upregulation of COX-2 is needed to synthesize PGE_2 to refill the intracellular stores; (3) arachidonic acid precursor stores were depleted; (4) the flow stimulus was insufficient to cause cells to release PGE_2 at all (F. Pavalko, personal communication).

Rabbit tendon cells did not significantly increase intracellular calcium in response to a fluid-induced shear stress of up to 25 dyn/cm^2. Either these cells do not have a mechano-sensitive channel that allows calcium entry in response to a mechanical stimulus, or the mechanical stimulus offered by fluid flow is not sufficient to activate these channels. Since these same cells significantly increased $[Ca^{2+}]_{ic}$ in response to a poke stimulus, a phenomenon that also requires influx of extracellular calcium (Sanderson et al., 1990), it is more likely that the flow stimulus did not activate a channel that allows the entry of extracellular calcium.

Cells from other musculoskeletal tissues have been shown to increase intracellular calcium levels by 25–140% in response to fluid-induced shear stress (Gupta et al., 1998; Hung et al., 1995, 1997; Yellowley et al., 1997). In comparison, rabbit tendon cells had a peak increase of 20% when exposed to 10 dyn/cm^2, and 71% when exposed to 25 dyn/cm^2. Changes in intracellular calcium are only one way in which cells respond initially to a mechanical or biochemical stimulus (Banes et al., 1995). The results of the present experiment show that a significant increase in intracellular calcium is not necessary for gene expression to be altered in response to fluid flow. Others have shown that a pathway involving G-protein receptors in the cell membrane may be activated by shear stress without the need for calcium mobilization (Berk et al., 1995; Hung et al., 2000). It is also possible that the observed increases in $[Ca^{2+}]_{ic}$, although not statistically significant, could be "biologically significant", and sufficient to trigger downstream events such as changes in gene expression.

The fact that we did not observe a substantial calcium transient does not exclude the role of Ca^{2+} in the response to fluid flow—it only indicates that these events are not coupled in rabbit tendon cells. It has been recently reported that Ca^{2+} regulates fluid-induced cytoskeletal reorganization and gene expression in osteoblasts (Sakai et al., 1998; Chen et al., 2000). One of the limitations of our study is that we did not use calcium channel inhibitors or blockers of intracellular calcium release during our experiments. These would have been necessary to directly assess the role of calcium on the observed response to fluid flow. Another limitation of our study is that we could not match the levels of fluid shear stress in these experiments to those that occur in vivo, since these are unknown.

In our experiments, upregulation of COX-2, IL-1β, MMP-1 and MMP-3 genes occurred in response to fluid flow without an associated calcium transient, suggesting that other pathways are involved in the mechanocoupling of this signal. Overall, these results demonstrate the potential of rabbit tendon cells to respond to fluid flow by producing factors, such as cyclooxygenase II and matrix metalloproteinases, that may potentially contribute to inflammation and degeneration of tendons in vivo.

Acknowledgements

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