Pifithrin-α, an inhibitor of p53 transactivation, alters the inflammatory process and delays tendon healing following acute injury

David Marsolais,1,3 Claude H. Côté,1,2 and Jérôme Frenette1,2

1CRML, CHUL Research Center and 2Department of Rehabilitation, Faculty of Medicine, Université Laval, Quebec City, Quebec, Canada; and 3The Scripps Research Institute, La Jolla, California

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Marsolais D, Côté CH, Frenette J. Pifithrin-α, an inhibitor of p53 transactivation, alters the inflammatory process and delays tendon healing following acute injury. Am J Physiol Regul Integr Comp Physiol 292: R321–R327, 2007. First published August 10, 2006; doi:10.1152/ajpregu.00411.2005.—Transcription factor p53, which was initially associated with cancer, has now emerged as an important regulator of inflammation and extracellular matrix homeostasis, two processes highly relevant to tendon repair. The goal of this study was to evaluate the effect of a p53 transactivation inhibitor, namely, pifithrin-α, on the pathophysiological sequence following collagenase-induced tendon injury. Administration of pifithrin-α during the inflammatory phase reduced the accumulation of neutrophils and macrophages by 30 and 40%, respectively, on day 3 postinjury. Pifithrin-α failed to reduce the percentage of apoptotic cells following collagenase injection but delayed functional recovery. In uninjured Achilles tendons, pifithrin-α increased metalloproteinase activity 2.4-fold. Accordingly, pifithrin-α reduced the collagen content in intact tendons as well as in injured tendons 7 days posttrauma compared with placebo. The effect of pifithrin-α on load to failure and stiffness was also evaluated. The administration of pifithrin-α during the inflammatory phase did not significantly decrease the functional deficit 3 days posttrauma. More importantly, load to failure and stiffness were significantly decreased in the pifithrin-α group from day 7 to day 28 compared with placebo. Overall, our results suggest that administration of pifithrin-α alters the inflammatory process and delays tendon healing. The present findings also support the concept that p53 can regulate extracellular matrix homeostasis in vivo.

inflammation; collagen; apoptosis

Tendon healing can take several years following an acute trauma (37). Prolonged alteration of tendons is associated with muscular atrophy, accumulation of intramuscular connective tissue, formation of adherences, and persistent reduction of functional capacity (15), which ultimately has an impact on the quality of life and health care expenses (5). The socioeconomic costs of tendon injuries have progressed steadily over the last 20 years, presumably as a consequence of our contemporary lifestyle (14). To date, there are few or no therapies that significantly accelerate tendon healing. However, it is becoming evident that several mediators of inflammation can favor healing, and a better understanding of the interplay between these mediators during tendon repair may provide a solid foundation for developing more effective treatments.

Inflammation is a tightly regulated multistep process that is essential for the prevention of infection, removal of debris, and initiation of the healing response. Vasodilation, activation of the endothelium, and establishment of a chemotactic gradient favor the recruitment of neutrophils and macrophages to the site of the injury (32, 33). These subsets of leukocytes may produce reactive oxygen and nitrogen species and induce intact cell death, presumably by necrosis and p53-dependent apoptosis (24). Inflammatory cells also release a variety of matrix metalloproteases (MMPs) that are thought to damage the extracellular matrix (ECM) (18). After this catabolic phase, macrophages engulf apoptotic neutrophils and release different growth factors such as transforming growth factor, basic fibroblast growth factor, vascular endothelial growth factor, and MMPs that are essential for later initiation of ECM deposition and remodeling (26). In this particular case, leukocyte apoptosis would be an essential step for the resolution of inflammation and initiation of healing. Recent advances in the fields of oncology and pathology strongly suggest that transcription factor p53, which is a powerful tumor suppressor and apoptotic promoter (35), also may be an important mediator of inflammation and ECM homeostasis. Moreover, p53 was shown to be upregulated following tendon injury (16). In view of these evidences, we hypothesized that p53 plays a central role in the repair process that follows an acute tendon trauma.

In the present study, we employed an in vivo model to gain a better understanding of the role of p53 in tendon homeostasis and repair. We hypothesized that the administration of pifithrin-α (PFT), an inhibitor of p53 transactivation, during the inflammatory phase would 1) reduce the accumulation of inflammatory cells, 2) inhibit caspase-3-dependent apoptosis, and 3) ultimately accelerate tendon healing. Our results indicate that the administration of PFT during the inflammatory phase reduced the accumulation of leukocytes following collagenase-induced injury. Unexpectedly, PFT diminished collagen content and retarded the onset of healing in Achilles tendons.

MATERIALS AND METHODS

Tendon injury. The animals were cared for and handled according to the Canadian Council of Animal Protection guidelines, and all protocols were approved by an independent review committee. Female Wistar rats (Charles River) weighing 180–200 g were used for these experiments. Upon arrival, the rats underwent a 5-day acclimatization period and were paired in standard 20 40-cm cages. They were allowed normal cage activity and provided with chow and water ad libitum. Surgical procedures for the induction of tendon injury were conducted as previously described (20). Briefly, anesthesia was performed with an intraperitoneal injection of ketamine-xylazine (87.5 and 12.5 mg/kg, respectively), after which 5 ml of lactated Ringer was injected subcutaneously to prevent dehydration. The rats...
were injected with 30 µl of crude collagenase (Sigma) in both Achilles tendons at a concentration of 10 mg/ml in sterile phosphate-buffered saline (PBS). This concentration of collagenase causes a reversible tendon degeneration accompanied by a classic inflammatory response. The animals were then killed at various time points by cervical dislocation under anesthesia for mechanical testing or by CO2 inhalation for histological analyses.

Drug administration. To inhibit p53 transactivation during the inflammatory phase, we injected 2.2 mg/kg PFT (Alexis Biochemicals) intraperitoneally at 24 h intervals for 5 days starting 25 h before the induction of acute tendon injury. The placebo (PCB) group received identical volumes of the vehicle, that is, 1 µl per gram of body weight of an aqueous solution containing 0.9% sodium chloride and 4.4% dimethyl sulfoxide. This dose of PFT was shown to exert protective and anti-inflammatory effects in various models (34).

Mechanical testing. To measure functional properties, we excised the Achilles tendons with the calcaneus and the inferior portion of the triceps surae. The muscle tissue was gently removed from the aponeurosis, and the tendons were installed on a MTS 858 Mini Bionix II device (MTS Systems) consisting of a hydraulically driven linear voltage differential transformer connected to a 0.5-kN load cell. The initial length (l₀) was manually set at a tensional force of 1–2 N. Tension-elongation curves were performed using a strain rate of 10% of l₀ per second until rupture. Tension and elongation were monitored at a frequency of 10 Hz, and curves were plotted using Excel software (Microsoft). Specimens were discarded if slippage or absence of a clear rupture point was observed. Stiffness was defined as the maximal slope of the linear portion of the tension-elongation curve in a time lapse of 0.5 s. Ultimate rupture force and stiffness were also recorded (21).

Hydroxyproline determination. The Achilles tendons were dehydrated for 24 h at 60°C to determine their dry mass (31). They were then hydrolyzed in 6 N HCl at 130°C for 3 h, neutralized with NaOH, and diluted in water. The concentration of hydroxyproline in the tendon samples was determined as previously described (21, 36). Briefly, tendon extracts were oxidized with chloramine-T, the reaction was stopped using perchloric acid, and samples were incubated with Ehrlich’s reagent at 60°C for 20 min. After cooling, absorbance was read at 550-nm wavelength. All samples were processed simultaneously, and each sample was assayed in triplicate.

Histology. After death, rat hindlimbs were immediately removed, immobilized in a dorsiflexion position, and submerged in a zinc-based fixing solution for up to 48 h (21). Thereafter, the tendons were isolated, submitted to routine histological processing, and embedded in paraffin (21). Two random longitudinal sections were cut and immunolabeled for neutrophils (anti-neutrophil; Accurate Chemical), macrophages (anti-CD68; Serotec), or apoptotic cells (anti-human/mouse activated caspase-3; R&D Systems). To evaluate the density of the neutrophils and macrophages, the cells were counted and expressed as a function of the volume of tissue examined, as previously described (3, 20, 21). To determine the percentage of apoptotic cells, tissue sections were immunolabeled for activated caspase-3 and counterstained with hematoxylin. Total activated caspase-3-positive cells and nuclei were then counted manually under bright-field microscopy, and the number of positive cells was expressed as a percentage of nuclei.

In another series of experiments, tendons were frozen in Tissue-Tek OCT medium, and longitudinal cryosections were performed at a 10-µm thickness. Sections were fixed in PBS containing 4% formaldehyde, blocked as previously described (20), and overlaid with different cocktails of antibodies. To determine the nature of cells undergoing apoptosis at the site of injury, we simultaneously incubated tendons with mouse anti-CD43 (Serotec), mouse anti-CD68, and rabbit anti-human/mouse activated caspase-3. After being washed, slides were incubated with Alexa Fluor 546-coupled anti-rabbit IgG (Molecular Probes) and then Alexa Fluor 488-coupled anti-mouse IgG (Molecular Probes). To determine the distribution of p53, we overlaid tendon cryosections with rabbit anti-p53 (Cell Signaling Technology) and, sequentially, with biotinylated anti-rabbit IgG (BD Pharmingen) and FITC-coupled streptavidin (BD Pharmingen). Labeling was visualized using a Bio-Rad Radiance 2100 Rainbow laser scanning system attached to a Nikon TE2000-U microscope. Images were acquired serially and reconstructed using Imaris software (Bitplane).

Gelatin zymography. Tendons were homogenized on ice in non-reducing sample buffer containing 10% glycerol, 2% SDS, and 0.5M Tris, pH 6.5. Gelatin zymography was performed by submitting equal amounts of proteins to a SDS-PAGE in 9% polyacrylamide gel containing 1 mg/ml gelatin. After migration, gels were washed in 2.5% Triton X-100 to remove SDS and allow renaturation of proteins. Gels were then washed three times with digestion buffer consisting of 50 mM Tris (pH 7.5), 5 mM CaCl, and 1 mM ZnCl and were incubated for at least 4 h at 37°C. Gels were stained in 0.25% Coomassie brilliant blue R250 (Bio-Rad) and destained in Coomassie blue solvent. Lysis bands were quantified using MetaMorph software (Universal Imaging) (25).

Fig. 1. Pifithrin-α (PFT) reduced the accumulation of inflammatory cells following collagenase-induced tendon injury. Neutrophil (A) and macrophage (B) concentrations were quantified in ambulatory control animals (CTR) that received 5 doses of placebo (PCB) or PFT. Tendons also were examined 3, 7, 14, and 28 days after the collagenase injection. Data are means ± SE of 5–7 animals. #Significantly different from the ambulatory control. *Significantly different from the PCB. P < 0.05 unless otherwise stated.
Statistical analysis. Concentration of inflammatory cells and mechanical properties were assessed using two-way analysis of variance (ANOVA). Afterwards, Scheffé’s tests were performed to compare means when a significant $F$ ratio was obtained. Where applicable, unpaired Student’s $t$-test also was performed. Finally, we performed one-way ANOVA to evaluate the separate effect of PFT or PCB as a function of time, regarding the different parameters investigated. All tests were carried out in a bilateral fashion, and a $P$ value $<0.05$ was considered to be significant, unless otherwise specified. All data are presented as means ± SE.

Fig. 2. The percentage of activated caspase-3-positive cells was not affected by PFT following acute tendon injury. Tendons were immunolabeled with anti-activated caspase-3 following collagenase injection. Omission of the primary antibody produced no labeling (A). No positive cells were detected in tendons harvested from ambulatory control animals (B). However, several activated caspase-3-positive cells were detected 3 days after the collagenase injection (C). The percentage of positive cells was not affected by the administration of PFT compared with the PCB (D). Data are means ± SE of 5 or 6 animals. $P < 0.05$.

Fig. 3. Inflammatory and noninflammatory cells undergo apoptosis after collagenase-induced tendon injury. Three days following collagenase injection, rounded nuclei cells (A) expressing inflammatory cell markers for neutrophils and/or macrophages (green; B) as well as activated caspase-3 (red; C) were detected (D shows merged image of A–C). CD43 $^+$ CD68 $^+$ cells located between collagen fibers also were found to contain activated caspase-3 (E). Tendons harvested from ambulatory control animals did not contain detectable amounts of p53 (F). Cells positive for p53 (green) could be found 3 days (G) and 5 days (H) following collagenase injection.
RESULTS

Inflammatory cell accumulation. The observed sequence and kinetics of leukocyte accumulation were consistent with those that have been previously reported (3, 20). Injection of collagenase into the Achilles tendon induced a significant accumulation of neutrophils and macrophages that peaked on day 3 and decreased thereafter (Fig. 1). Neutrophil and macrophage densities returned to control values by days 14 and 28, respectively. Compared with PCB animals, PFT-treated animals had significant lower leukocyte numbers. With respect to specific subpopulations, PFT animals had 30 and 40% lower neutrophil and macrophage numbers, respectively, at day 3 following injury. The concentrations of both subsets of inflammatory cells in the PFT and PCB animals were identical from day 7 until the end of the time course. The administration of five doses of PFT did not change the concentrations of neutrophils and macrophages in ambulatory control animals.

Influence of PFT on apoptosis. To determine whether the administration of PFT influences apoptotic cell death, we compared percentages of activated caspase-3-positive cells in PCB and PFT-treated animals at days 3 and 5 following tendon injury. We used these time points because 1) apoptosis is known to peak when the accumulation of inflammatory cells begins to decline, and 2) we also intended to evaluate apoptosis shortly after the end of PFT treatment. Activated caspase-3-positive cells were not detectable into control tendons (Fig. 2B) but were detected following collagenase injection (Fig. 2C) and constituted ~1.2% of the total cells. The percentage of immunoreactive cells for activated caspase-3 was not different between PFT and PCB-treated animals 3 and 5 days following collagenase injection (Fig. 2D). Immunofluorescence analyses showed that both inflammatory cells (Fig. 3, A–D) and tendon cells (Fig. 3E), presumably tenocytes/tenoblasts, were undergoing cell death 3 days following collagenase injection. p53 could not be detected by immunofluorescence in control tendons (Fig. 3F), but immunoreactive cells were detected 3 and 5 days following collagenase injection (Fig. 3, G and H).
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respectively). Together, these results showed that p53 accumulated into cells and that the rate of apoptosis was increased, but PFT treatment did not influence the number of activated caspase-3-positive cells following tendon trauma.

Influence of PFT on collagen content and biomechanical properties. The administration of PFT did not significantly attenuate the functional defect during the inflammatory phase (Fig. 4). Collagenase injection induced a 50% decrease in load to failure at day 3 compared with ambulatory control animals. The maximal reduction of load to failure was delayed and occurred around day 7 in PFT-treated animals. Moreover, the PCB group returned to control values 14 days earlier than the PFT group. Load to failure and stiffness were significantly lower from day 7 to day 28 in the PFT-treated animals compared with PCB. These results suggest that there was a delay in the onset of the recovery phase in animals treated with PFT. These results are supported by the observations that PFT significantly reduced collagen content in injured and intact tendons (Fig. 5A) and that MMP activity was increased in tendons excised from control animals treated with PFT for 5 days relative to the PCB (Fig. 5B).

DISCUSSION

p53 is involved in the pathophysiology of various acute conditions such as skin wounds (34), burns (23), cerebral ischemia (19), hepatic (27) and renal injuries (6), and liver transplant (8). New evidences support the concept that p53 is involved in the pathophysiology of various acute conditions. Inflammation and healing follow tendon injury, such as tenoblasts and resident macrophages, release different matrix metalloproteinases (MMPs) whose interactions influence ECM homeostasis.

PFT delays tendon recovery. Our result also showed that PFT delayed the onset of tendon repair. This observation was at first glance unexpected and led us to test whether PFT administration would favor collagen catabolism. Administration of PFT delayed the onset of tendon repair. This observation was at first glance unexpected and led us to test whether PFT administration would favor collagen catabolism. Administration of PFT delayed the onset of tendon repair. This observation was at first glance unexpected and led us to test whether PFT administration would favor collagen catabolism.
tion of PFT to control animals was found to increase the gelatinolytic activity and reduce the amount of hydroxyproline in Achilles tendons. Because the cellular content of p53 is very low under normal conditions in tendons, PFT may have modulated collagen homeostasis by p53-independent mechanisms. Indeed, PFT was shown to alter heat shock proteins signaling pathways (17), which are known to be involved at precise steps of collagen synthesis (13). On the other hand, overexpression of p53 also can result in a major reduction in the production of proMMP-13 (collagenase-3) (30) and MMP-1 (collagenase-1) (1). In addition to these intracellular events, the inhibition of p53 transactivation also may alter collagen content during the healing phase by perturbing the inflammatory process. Indeed, neutrophils and macrophages can play deleterious roles (7) or promote tissue repair by phagocytosing cellular debris and releasing essential cytokines for tissue healing (9, 22). The beneficial role of macrophages is further supported by our findings that a reduction of neutrophil and macrophage concentrations in PFT-treated animals would delay both the maximal functional loss and the recovery period. Thus the inhibition of the inflammatory process by PFT during the catabolic phase would interfere with the initial steps of healing rather than alleviating nonspecific damage to the intact ECM.

Strengths and weaknesses of the experimental model. In this study, we used a well-characterized model in which collagenase is injected into rat Achilles tendon. One of the main advantages of using this model is that no immobilization under cast is required so that we can study in parallel the involvement of inflammatory cells and mechanical loading during tissue healing. It is also relatively noninvasive, easy to perform, reproducible, and well validated. However, the collagenase model also presents some disadvantages. Crude collagenase is a foreign body that may induce a specific cellular response by itself. Moreover, the presence of an exogenous protease may influence the regulation of MMPs and tissue inhibitors of metalloproteinases. As a whole, collagenase-induced tendon injury is an artificial but useful model that allows study of the interplay among the inflammatory cells, mechanical loading, and tissue healing.

In conclusion, the regulation of tendon pathophysiology is still poorly understood, and no therapeutic strategy has yet proven to be really effective in accelerating tendon healing. Our results show that p53 accumulates after tendon trauma and that inflammatory cells, as well as tendon cells, undergo apoptosis in the process. Our findings indicate that PFT alleviates inflammatory cell accumulation following collagenase-induced tendon injury, increases collagen catabolism, and delays tendon healing. Although PFT may perturb cellular events others than p53 transactivation, such as kinase activity or glucocorticoid receptor signaling, our work and work from others indicate that p53 plays a role in the regulation of ECM homeostasis and inflammatory response following an acute trauma (Fig. 6). These results should at least improve our understanding of the regulation of tendon healing and may help to delineate future therapeutic strategies to optimize recovery following acute or chronic tendon pathology.

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REFERENCES


