Relationship between Apoptotic Response in the Tendon and Initial Mechanical Parameters after Fatigue Loading

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INTRODUCTION: Tendinopathy leading to tendon rupture is common and attributable to damage accumulation from repetitive use. Despite its high incidence, the biological mechanisms responsible for the initiation and progression of tendinopathy are poorly understood. Several studies suggest that apoptosis may be implicated in the degenerative process of tendinopathy. Using our in vivo rat patellar tendon model of fatigue damage accumulation, we previously showed greater apoptotic activity for moderately fatigue loaded (7200 cycles) compared to control tendons at 7 and 14 days post fatigue loading. We have also shown that precise control over the input parameters to induce damage in our model, natural variation among tendons can result in varying amounts of induced damage for the same loading protocol, leading us to identify initial mechanical parameters that are indicative of the damage induced. Assessing the temporal response of the tendon in the context of both the magnitude of induced damage and apoptotic activity 3 days after fatigue loading. We hypothesized that greater apoptotic activity will be associated with greater induced damage.

METHODS: Following IACUC approval, left patellar tendons of anesthetized retired breeder female Sprague-Dawley rats were surgically exposed. The tibia was fixed at ~30° knee flexion and the patella was gripped with a clamp that was connected to an Instron testing machine to allow for fatigue loading. Rats were fatigue loaded under load control (1N-40N range) for either 100 (n=6) or 7200 (n=8) cycles at 1 Hz. Diagnostic tests (1N-15N) were applied before (diag1: 420 cycles) and after (diag2: 120 cycles) fatigue loading. As previously described, the effect of fatigue loading on initial damage indices, including hysteresis, slope of the loading and unloading load-displacement curves (loads and unloading stiffness, respectively), and elongation, was determined by comparing these parameters between the last 10 cycles of diag1 and diag2. After loading, skin was sutured, analgesia administered and rats resumed cage activity. Additional rats (n=6) were used as naïve controls.

Rats were sacrificed 3 days after fatigue loading. The quadriceps-patella-patellar tendon-tibia complex was harvested and fixed in zinc buffered formalin under a –2N tensile load. Tissue blocks were decalcified, embedded in paraffin and sectioned at 5 μm. Immunohistochemical staining for cleaved Caspase-3 was used to identify apoptotic cells. Sections were incubated in a 1: antibody against cleaved caspase-3 (Cell Signaling). Detection was performed using a HRP-polymer labeled 2 antibody with DAB enhancement. Sections were counterstained with methylene blue to highlight negative staining control.

Under 400X magnification, normal and apoptotic cells (Caspase3+) were counted at the insertion (tibial end), origin (patellar end) and mid-substance (Fig 1) and the percent apoptotic cells was determined for the pooled tendon and for each region. Data were collected by a single individual blinded to specimen identity. Apoptosis between control and loaded tendons and between cycle groups was compared using t-tests. Differences among regions within each group were assessed with repeated measures ANOVA. Correlations between initial damage parameters and 3-day apoptotic activity were assessed. Non-linear relationships were identified with k-means cluster analysis with 2 clusters as outcomes were expected (low and high damage). An initial damage parameter was dismissed if a resulting cluster contained less than 3 data points. Differences between clusters were tested by Mann Whitney tests and significance (*) reported at p<0.05.

RESULTS: Apoptotic activity 3 days after fatigue loading did not significantly increase with the number of loading cycles. Naïve tendons exhibited higher apoptotic activity at the insertion than midsubstance or origin. However, no significant regional differences were observed for the 100 and 7200 cycle groups.

In contrast, the apoptotic activity increased with increased changes in initial damage parameters. No correlations were found between 3-day apoptotic activity and any initial damage parameter for the pooled tendon or any region (data not shown). However, k-means cluster analysis showed significantly greater 3-day apoptotic activity in pooled tendons that exhibited greater increase in initial unloading stiffness (data not shown). Similarly, cluster analysis showed that initial hysteresis loss and increase in unloading stiffness (which were correlated) coincided with greater apoptotic activity in the midsubstance and insertion (Shown for unloading stiffness in Fig 3).

DISCUSSION: Our data supports our hypothesis that apoptosis is induced by accumulation of fatigue damage. While the number of fatigue loading cycles generally correlates with the initial damage parameters (previously shown)12, evaluating the response of the tendon in the context of these initial parameters accounts for natural variability between tendons that may result in a range of induced damage from the same number of loading cycles. We have previously shown that 7-day stiffness can be predicted by initial hysteresis loss and 7-day molecular response can be predicted by initial unloading stiffness and elongation13. In this study, we have found that changes in initial hysteresis and unloading stiffness are significantly related to the 3-day apoptotic activity. Taken together, our data suggests that changes in the tendon structure that are reflected by changes in hysteresis and unloading stiffness impact the 3-day apoptotic activity which may lead to the previously observed 7-day molecular and mechanical changes.

Apoptotic activity is increased in intact supraspinatus tendons with subacromial impingement1, in painful tendinopathy10 and in edges of torn tendons1, 2. These findings along with our data suggest that apoptosis is implicated from initiation to progression of tendinopathy in intact and eventually ruptured tendons. We expect that therapeutic interventions that target blocking apoptosis may be essential to inhibiting development of tendinopathy. Our pilot data shows that injecting rats with 2 intraperitoneal injections per day of the pan caspase inhibitor, Q-VD-OPh, results in downregulation of cleaved caspase-3, 3-days post fatigue loading (Fig 4). We will evaluate the long term effect of inhibiting apoptosis on the ability of fatigue damaged tendons to repair and heal.

SIGNIFICANCE: Our data demonstrates that apoptosis in the tendon is proportional to the amount of damage induced. Therapies focused on inhibition of apoptosis can be integral to management of tendinopathy.

ACKNOWLEDGEMENTS: AR52743 (ELF) and AR058123 (NAP) from NIAMS/NIH. The authors thank Stephen J. Ros and Michael R. Boniello for their contributions.