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Excessive Apoptosis in Patellar Tendinopathy in Athletes

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Background: The pathogenesis of tendon overuse injuries is poorly understood. The histopathology underlying tendinopathy at various anatomical locations is similar and may reflect a common pathologic process.

Hypothesis: Apoptosis contributes to the pathophysiology in patellar tendinopathy.

Study Design: Case control study; Level of evidence, 3.

Methods: We compared biopsy specimens from the patellar tendon in patients with patellar tendinopathy diagnosed clinically and with typical magnetic resonance image findings with biopsy specimens from a control group without any previous or current knee complaints to suggest patellar tendinopathy. The presence of apoptosis was examined with immunohistochemical methods using a polyclonal antibody recognizing active caspase-3, confirmed by labeling DNA strand breaks (F7-26 antibody) and nuclear morphology (fragmentation and condensation).

Results: The number of apoptotic cells per unit area (4.5 mm^2) was 0.91 ± 0.81 (SD) in tendinopathic samples and 0.21 ± 0.21 in controls (P = .026). Although the tendinopathic samples displayed increased cellularity (average 162.5 nuclei/mm^2 vs 98.9 nuclei/mm^2), the apoptotic index was higher (0.42% vs 0.17%, P = .014).

Conclusion: Increased apoptotic cell death is a feature of patellar tendinosis. The role of apoptosis within the broader framework and time course of tendon overuse injury remains to be established.

Keywords: tendon; patellar tendinosis; apoptosis

Overuse tendon injury—tendinopathy—is a common, recalcitrant problem in sports medicine. It has major effects on quality of life for competitive and recreational athletes.[4,25] Despite the currently available treatment options,[10,15,18,29,32] the condition can be career-ending.

Patellar tendinopathy is considered to result from tendon overload caused by internal and external factors. Regarding external factors, Ferretti et al[16] showed that there is a linear relationship between training volume and prevalence of tendinopathy among volleyball players, and that the harder the floor type on which they train, the higher the prevalence of patellar tendinopathy. In a recent epidemiological study among high-level athletes in sports with different quadriceps loading patterns,[25] the prevalence varied between sports—from no cases in cycling and orienteering to 32% and 44% with current symptoms in male basketball and volleyball, respectively.[25] It should also be noted that the mean duration of symptoms was more than 2 years, and that the affected athletes reported significant pain and reduced function levels.[25] Studies on internal pathogenic factors also indicate that athletes who subject the tendon to higher loads are at higher risk for tendinopathy.[26,28] However, the pathophysiological processes occurring within the tendon substance are mostly unknown, and there is a need for further studies.[1]

Tendons are characterized by a homeostatic balance, as in all other living tissues, with both inhibitory and stimulatory growth and survival signals.[8] It has been suggested that the earliest identifiable morphological changes in tendinosis occur in the tenocytes, not the collagen fibers.[11,44] One of the striking histological findings in biopsy specimens from tendinopathic tendons is the scarcity of inflammatory cells and a consistent histopathological picture featuring abnormal

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tenocyte shape and density, along with accumulation of glycosaminoglycans and collagen fiber thinning and disarray, with or without neurovascular proliferation.[11,19,21,22,30] Apoptosis, also called "programmed cell death," is a specific response to both physiological and stressful stimuli and is characterized by distinctive morphological and biochemical changes.[24,37] If the tenocyte is involved in the primary pathological changes, this may be compatible with a degenerative, apoptotic process.[44] Alternatively, apoptosis is commonly associated with late-stage remodeling of reparative tissue.[17,34]

In ruptured human rotator cuff specimens, Yuan and colleagues[43] reported excessive apoptosis using Terminal Deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling (TUNEL) and DNA laddering assays. The authors concluded that in this end-stage tendon disease in older individuals (average age 61 years), apoptosis was clearly present. Nevertheless, their study design did not allow them to determine whether apoptosis was present before or only after rupture. In addition, the effects of aging, anatomy, and vascular supply limit the ability to extrapolate to other tendons. Previous studies have shown that tenocytes undergo apoptosis in response to hypoxia, oxidative stress, or excessive tensile load.[35,36,42] Therefore, using athletes' patellar tendon tissue with clinical and morphological features of tendinosis, we sought to determine the presence and extent of apoptosis in symptomatic but not ruptured tendons of young, active athletes, using antibodies against activated caspase-3 and fragmented DNA.

METHODS

Patient Groups

The patient group (n = 23) included athletes from different sports who were included in a prospective randomized trial comparing surgery with eccentric training.[2] The following diagnostic criteria were used for patellar tendinopathy: history of exercise-related pain in the proximal patellar tendon or the patellar insertion and distinct tenderness to palpation corresponding to the painful area.[5] To be included in the study, patients had a clinical diagnosis of jumper's knee grade IIIB, that is, pain during and after activity and inability to participate in sports at the same level as before the injury.[26] In addition, to ensure that the biopsy specimens were taken from the tendinopathic area, a thickening and signal changes corresponding to the painful area on MRI were required. Finally, patients had to have experienced symptoms for a minimum of 3 months and be willing to undergo surgery.

Each patient went through a standardized interview, and the information requested from each athlete included age, height, weight, and number of years participating in organized athletic training. Patients were asked to report the number of training hours per week during the competition season (sport-specific training, weight training, jump training, and other types of training). To assess the severity of the condition, the athletes with diagnosed current patellar tendinopathy also self-recorded their symptoms and level of sports function using the Victorian Institute of Sport Assessment (VISA) questionnaire.[41] This brief questionnaire assesses symptoms, function, and the ability to play sports.[41] The maximal VISA score for an asymptomatic, fully performing individual is 100 points, and the theoretical minimum is 0.[41] The VISA questionnaire is a reliable and valid measure of symptoms in patients with patellar tendinopathy.[41]

The control group (n = 11) was selected from patients treated with marrow nailing for tibia fractures from low-energy trauma. These patients had no current or previous knee complaints indicative of patellar tendinopathy. Subjects in both groups had to be at least 18 years old (to ensure that the epiphyses were closed) and able to understand oral and written Norwegian.

Exclusion criteria in both groups were previous surgical treatment in or around the same knee, corticosteroid injections in or around the same knee, serious traumatic injury affecting the same knee, and any rheumatic or degenerative knee condition. The study was approved by the Regional Committee for Research Ethics, participation was voluntary, and written consent was obtained.

Surgical Technique

The surgical exposure was identical in the 2 groups, with a 5-cm longitudinal midline or lateral parapatellar incision, splitting of the paratenon, and exposure of the patellar ligament. The paratenon was split longitudinally; any pathologic paratenon tissue was removed, and the tendon was fully exposed. In both groups, biopsy specimens were taken from the proximal bone-ligament junction, and the tendon tissue was excised using a full-thickness wedge-shaped incision, being widest at the patellar pole and narrowing distally. In the patient group, all abnormal tissue was removed. If clearly abnormal tissue was not seen macroscopically, the excision was based on MRI signal changes. The lesion was located on the MRI scan, and the corresponding area was debrided during surgery. Typically, a wedge with a proximal base 1 cm wide and extending to an apex 2 to 3 cm distal from the patellar pole was removed. All biopsy specimens were taken from the proximal patellar tendon.

In the control group, biopsy samples were taken with a width of at least 5 mm and a length of at least 20 mm from the middle portion of the ligament starting at the bone-ligament junction. A suture was passed through the proximal end of the tendon to allow its identification during subsequent processing.

Biopsy Procedure

The handling of the biopsy specimens was identical in the 2 groups. Immediately after the surgical procedure, the specimens were transferred to Zamboni's solvent.[46] They were stored in this solution for 4 to 24 hours and then washed in 0.1 M phosphate-buffered NaCL, pH 7.2, with 15% sucrose (weight/volume) (PBS) and 0.1% natriumazide. This washing was done until the yellow color from the Zamboni solution could no longer be seen in the phosphate-buffered saline (PBS). The specimens were then stored in PBS at 4°C

for a minimum of 48 hours, after which they were embedded in paraffin.

Light Microscopic Appearance

Sections of 5-µm were routinely stained for hematoxylin and eosin (general morphologic characteristics) and Alcian Blue (sulphated glycosaminoglycans) and viewed at 100 to 630 magnification on a Zeiss Axioplan (Carl Zeiss Inc. Thornwood, NY) upright microscope. Areas of adipose or peritendinous tissue were avoided during subsequent analysis.

Detection of Apoptosis and Assessment of Caspase Activation

Apoptosis was assessed using a monoclonal antibody against single-stranded DNA breaks (F7-26; Chemicon, Temecula, USA) as described previously,[36] as well as with a polyclonal antibody against the active (cleaved) form of caspase-3 (Asp 175; Cell Signaling, Danvers, Ma) and propidium iodide staining (Sigma-Aldritch, St Louis, Mo) for nuclear shape. Of these methods, the cleaved caspase-3 antibody yielded the most specific and reproducible labeling of apoptotic cells in tonsil tissue (serving as a positive control) and was thus used for systematic quantification. The sections were cleared, quenched in 3% hydrogen peroxide, incubated in protein-free block for 15 min, then left overnight with the antibody diluted 1:50 in 0.1% bovine serum albumin in tris buffered saline (TBS). Slides were then sequentially exposed in a dark, moist chamber to horseradish peroxidase-conjugated goat-antirabbit (1:100, 30 min), fluorescyl-tyramide amplification reagent (DAKO Diagnostics, Glostrup, Denmark), antifluroescein-horseradish peroxidase, and finally 3,3´-diamino-benzidine (Vector Laboratories, Burlingame, USA) for 5 minutes (’), with 3 × 5’ washes in TBS between each step. Identically fixed and processed tonsil tissue, with or without the primary antibody, was used as positive or negative control, respectively.

Image Analysis

The identity of slides was masked with black tape. Using a 40× objective lens, the tissue section was illuminated with halogen or fluorescent light (488 nm wavelength), and respective areas of positive F7-26 or propidium iodide staining were captured at 1392 × 1045 pixels with a digital camera (Retiga Exi 1394, Qimaging Corp, Burnaby, Canada). For quantitation of apoptosis, 15 random areas (0.30 mm^2 each) from the proximal region were digitized. Cells were considered positive only if the labeling was intense and suggestive of apoptotic morphology.[30] A standard exposure time (50 ms) was used, and the contrast was not digitally adjusted.

Data Analysis

For quantitation of apoptosis, the following variables were compared using caspase-3 labeled tissue sections: total number of positive cells in all areas (x), total number of cells counted (y), Apoptotic Index ($x \div y * 100\%$), number of positive fields, and average number of positive cells per field. Results are presented as means with standard deviations (SD) unless otherwise noted. Comparisons between normal and tendinosis tissue were performed using the t test for independent samples (SPSS 13.0, SPSS Inc, Chicago, Ill, USA). Levene’s test for equality of variances was used with significance predetermined at $P = .05$, and the data were visually inspected to determine their normality.

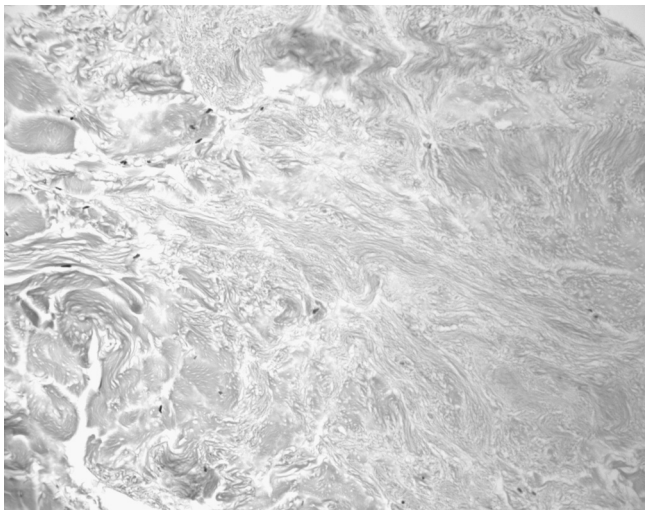

Figure 1. Patellar tendinosis specimen stained with hematoxylin & eosin, demonstrating a typical area of hypocellularity in association with collagen degeneration (100×). Note large area of absent cells in upper right.

RESULTS

Patient Characteristics

The mean age in the patient group was 30 years (24-34 years, n = 23) and in the control group, 29 years (19-43 years, n = 11). In the patient group, the mean number of years participating in organized training was 17 (10-28 years), and the mean number of total training hours per week was 14 (6-24 hours). The mean VISA score was 42 (15-65), and the mean duration of symptoms was 36 months (5-120 months).

Light Microscopic Appearance

Biopsy specimens from patients with a clinical history of tendon pain consistently revealed tendinosis, including areas of hypocellularity (Figure 1), as well as neovascularization with vessel wall thickening and collagen disarray and degeneration. Increased amounts of glycosaminoglycan were localized to areas of fibrocartilagenous metaplasia or to the tunica media of the vessel and perivascular regions. Inflammatory cells were virtually absent. Biopsy specimens from control tendons did not demonstrate any increase in vessel number or glycosaminoglycan content.

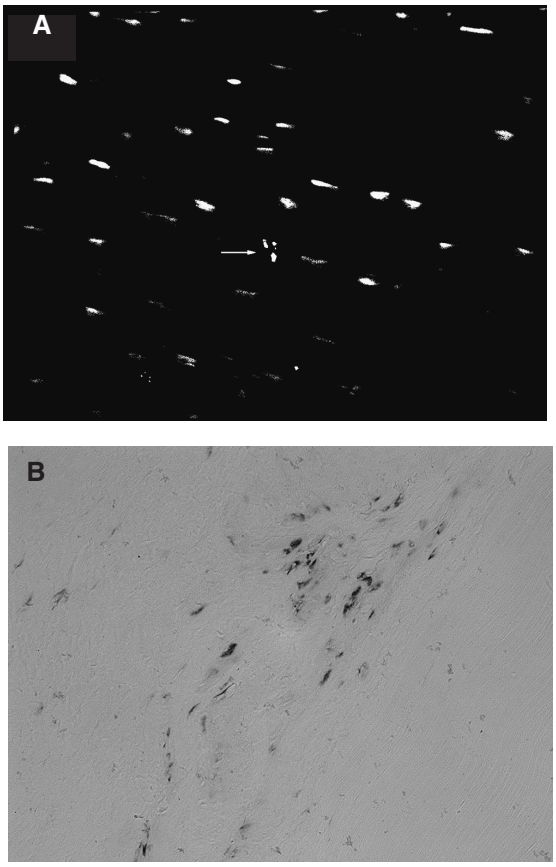

Figure 2. Demonstration of apoptosis in patellar tendinosis. A) Propidium iodide stain; arrow indicates fragmented nuclear shape (400×). B) F7-26 antibody demonstrating a cluster of apoptotic cells (200×).

Apoptosis in Normal and Overused Tendons

Apoptotic tenocytes were identified using all 3 methods (F7-26, propidium iodide, caspase-3) both in normal and pathological tendon, and each method showed that apoptosis represented a small minority of total cell counts (<1%). Apoptosis was predominantly found in fibroblast-like cells in the tendon proper (Figure 2A). Clusters of 5 to 10 apoptotic cells were observed in the tendinosis samples, compared with scattered or no cells in the controls (Figure 2B). Intense labeling with the caspase-3 antibody corresponded with nuclear fragmentation and condensation (Figure 3C). Faint cytoplasmic staining was sometimes observed and was interpreted as nonspecific (Figure 3D).

The number of apoptotic cells per unit area (4.5 mm^2) was 0.91 ± 0.81 in tendinosis samples and 0.21 ± 0.21 in controls (P = .026). There were more areas with apoptotic cells in the tendinosis tissue (3.7 ± 0.20 vs 1.9 ± 1.2, P = .006). In addition, fields from tendinosis patients that displayed positive staining had more apoptotic cells than positive fields from controls (3.1 ± 2.2 vs 1.5 ± 0.89, P = .006). Although the tendinosis samples displayed increased numbers of fibroblastic cells (162.5 ± 100 nuclei/mm^2 vs 98.9 ± 50 nuclei/mm^2, P = .021), the apoptotic index was higher (0.42 ± 0.38 % vs 0.17 ± 0.16 %, P = .014).

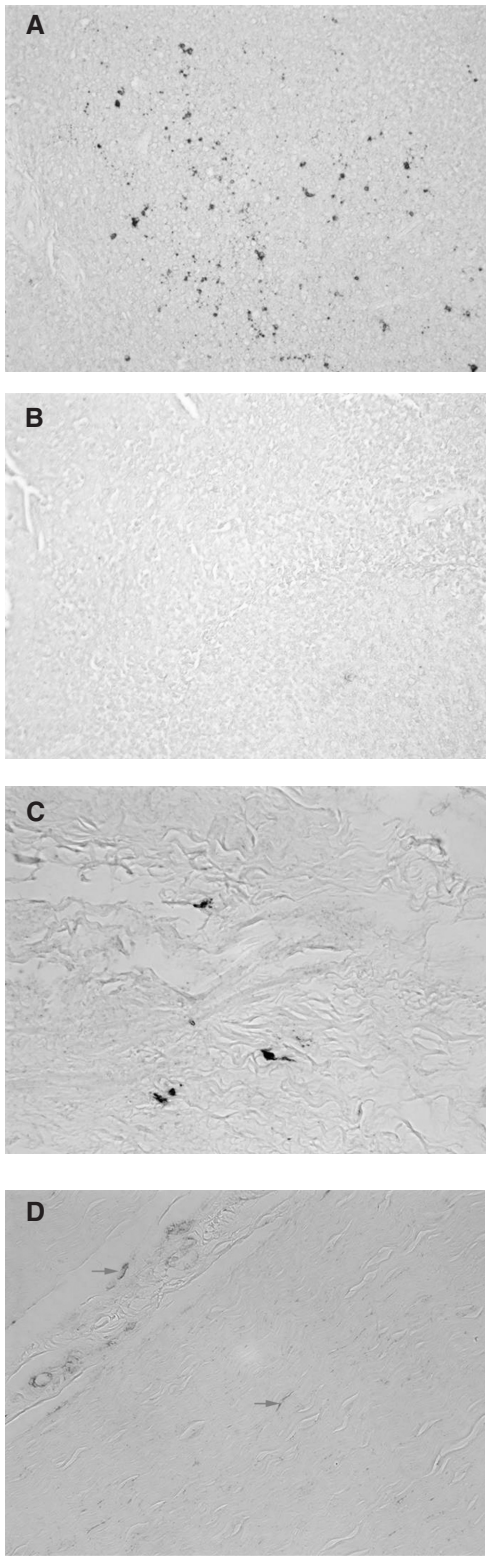

Figure 3. Demonstration of caspase activation in Bouin's fixed tissue. A) Positive staining for cleaved caspase-3 antibody coincides with apoptotic morphology in human tonsil. B) Omission of primary antibody yields no staining (100×). C) Intense labeling of tenocytes with fragmented morphology (200×). D) Arrows indicate background staining around vessels using the same conditions (200×).

DISCUSSION

The main findings in the present study were a significantly higher number of apoptotic cells per unit area and a significantly higher apoptotic index in biopsy specimens from the patellar tendons in patients with patellar tendinopathy compared with controls. Apoptotic tenocytes were identified using 3 methods: F7-26, propidium iodide, and caspase-3. The caspases are members of a family of cysteine proteases with a sequential activation and amplification system eventually causing apoptosis.[33] Since caspase-3 is one of the terminal proteins in the caspase activation system,[6,39] this finding denotes increased apoptotic activity in the patellar tendon in patients with patellar tendinopathy compared with controls.

To our knowledge, only 1 previous study has reported excessive apoptosis in the painful, nonruptured tendon.[40] That study used TUNEL staining, as well as the F7-26 antibody, to reveal apoptosis in the watershed area of the supraspinatus tendon with impingement. We note that apoptosis has also been seen in more severe tendon injuries, for example, tendon rupture.[43] How early in the pathogenesis of tendinosis apoptosis arises remains an important question.

The link between mechanical loading conditions and the pathophysiological response in tendinosis/tendinopathy is obscure, and currently there is insufficient evidence to provide a direct explanation for the possible connection between the loading pattern and the in vivo pathological response.[36] We have shown in case control[27] and cohort[28] studies that volleyball players with jumper's knee have better jumping ability and power generation than players who do not report symptoms from their tendons, presumably because they subject their knee extensors to higher loads when jumping and landing. Also, prevalence is higher in sports that require frequent jumping[25] and among athletes who train more.[16] Thus, there is reason to believe that there is a connection between the tendon-loading pattern and the pathologic changes within the tendon substance. In a study by Yuan et al,[43] excessive apoptosis was observed at the edge of torn rotator cuff tendons in elderly patients compared with controls. This has led to the proposal that tendinosis may begin as a degenerative process involving tenocyte death.[44] In support of this model, Skutek et al[39] suggested that mechanical stretching of tendon fibroblasts activates cell signaling pathways leading to apoptosis. However, once ruptured, the supraspinatus tendon would likely not receive excessive tensile loads, therefore other mechanisms such as oxidative stress, hypoxia, or remodelling may predominate in later stages. In another study, Barkhausen et al[3] found that different repetitive cyclic longitudinal stress patterns resulted in different cellular reactions depending on the strength of the applied stress. Repetitive stress applied during 1 day stimulated both proliferation and apoptosis.[3] In the current study, the number of tenocytes was increased overall, but there were also discrete areas of apoptosis and hypocellularity, suggesting that death and proliferation may be occurring simultaneously in response to repetitive loading, similar to the finding by Barkhausen et al.[3]

To successfully identify and quantify apoptotic cells in fixed tendon tissue, we used a well-characterized antibody recognizing active caspase-3, a key enzyme in the final common apoptotic pathway. This method resulted in specific labeling of cells with fragmented and condensed nuclei (Figure 3), which were found to compose <1% of the cell population in both patients and controls. This contrasts with a prior study of the patellar tendon[9] that reported surprisingly high rates of apoptosis in healthy controls (35% in rounded tenocytes and 26% in elongated tenocytes). It is not entirely clear why this discrepancy exists, but it likely relates to the use of the TUNEL technique, which has been criticized as generating many false-positive results.[24] Thus, the current paper presents a revised evaluation of the extent of tenocyte apoptosis in normal and painful patellar tendon.

A methodological limitation that must be considered when interpreting the results is that the diagnosis was based on clinical examination combined with MRI findings compatible with tendinosis. This means that to be recorded as having current symptoms of tendinopathy, the athlete had to report a painful tendon during athletic activity with corresponding palpation tenderness. It may be argued that this definition is nonspecific, since we do not know for certain that the tendon was the source of the pain in all cases. For instance, we could not rule out cases with referred pain, principally from the distal aspects of the articular surface of the patella. In fact, a number of studies have shown that the correlation between clinical findings and ultrasound[13,19,22,28,31] or MR examinations[7,14,22] is low, and even that symptoms and tendon changes come and go independently.[12,13] A significant number of athletes have or develop visible tendon changes without symptoms of jumper's knee, and some have significant pain without detectable tendon changes.[12,27] However, a diagnostic procedure based on MRI findings combined with a typical history and clinical findings is the most precise diagnosis of patellar tendinopathy currently in use. All athletes in the patient group had MRI findings localized to the proximal part of the patellar ligament, corresponding to the area where the biopsy samples were taken from. Thus, we would argue that the current diagnostic procedure is valid with regard to diagnostic precision and biopsy location. This is supported by the fact that the patients had an average symptom duration of 3 years and a VISA score of 42, which indicates a high level of pain and a significantly disabled patient group with chronic and severe complaints of patellar tendinopathy.

Our study contributes a novel insight toward understanding the pathologic changes associated with tendinosis, namely, that there is evidence of increased apoptosis in association with degenerate, nonruptured tendon compared with controls. However, there remain several limitations that must temper conclusions from our study. First, our cross-sectional study sheds no light on whether apoptosis preceded or followed the development of tendinosis. Second, our data were obtained in young athletes with chronic patellar tendinosis; we may not extend our conclusions beyond this population as yet. These limitations encourage future studies in other symptomatic or asymptomatic

tendons with early tendinosis, but it is a challenge to obtain such biopsy samples.

CONCLUSION

Our study demonstrates an increase in the extent of tendon cell apoptosis in athletes with patellar tendinopathy. The role of apoptosis within the broader framework and time course of tendon overuse injury remains to be established.

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