

Tenocyte apoptosis in the torn rotator cuff: a primary or secondary pathological event?

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ABSTRACT

Little information exists on the contribution of apoptosis to pathological tendon changes in rotator cuff tendinopathy. The purpose of this study was to quantitate the rate of tenocyte apoptosis in torn supraspinatus tendons and in the matched intact subscapularis and to examine the potential relation between apoptotic index (AI) and tendon pathology. In addition, the authors examined tenocyte density, proliferation rate and p53 gene expression patterns to gain further insight into relevant pathological mechanisms in the torn supraspinatus tendons with matched intact subscapularis tendon samples and 10 reference subscapularis samples were collected. Immunohistochemistry was used to define the AI (F7-26), proliferation rate (Ki67) and presence of p53 (M7001). Tendon degeneration was evaluated according to the Bonar scale. Expression of p53 and relevant genes (n=84) was examined on a subset of samples using microfluidic arrays. The AI was significantly increased in torn supraspinatus tendon and matched subscapularis tendon ($R^2=0.5742$; $p=0.0005$). Cell density and proliferation rate were also elevated in torn supraspinatus compared with reference subscapularis tendons ($p<0.05$). A significant increase in p53 occurred specifically in torn supraspinatus tendon ($p<0.05$), and several genes encoding p53-inhibiting proteins were downregulated in association, including HDAC1 ($p<0.05$), MDM4 ($p<0.001$) and PPM1D ($p<0.05$). Our results suggest that tenocyte apoptosis results from more than one mechanism in the injured rotator cuff, including both intrinsic factors related specifically to the torn supraspinatus tendon, as well as a more generalised effect on the adjacent subscapularis tendon.

INTRODUCTION

Rotator cuff injury ranks among the most prevalent of shoulder disorders causing pain and impaired function.¹ The incidence of tendon injury is rising due to an ageing population and increasing participation in recreational and sporting activities.^{2–4} Despite improved surgical techniques, re-tear rates are high.^{5–14} The poor healing capacity of the rotator cuff has sparked studies of degenerative mechanisms, including tenocyte apoptosis, which could potentially be minimised with biologic treatments at the time of repair.

Apoptosis is a regulated programme of cell death that plays essential roles in development and homeostasis. Apoptosis ensures appropriate cell density in adult tissues and the clearance of inflammatory and reparative cells during healing.^{15–17} Excessive apoptosis is associated with degenerative pathologies including

Alzheimer's disease,¹⁸ osteoarthritis^{19–22} and tendinopathy.^{23–31} Inflammation, infection, nicotine, hypoxia and mechanical injury are among the factors that may lead to increased apoptotic cell death.

Tenocytes play a pivotal role in maintaining a biomechanically competent extracellular matrix. These cells are typically defined by their elongated appearance, their organisation into longitudinal arrays and their high level of type I collagen synthesis. Tenocytes are typically quiescent, non-dividing cells. Areas of increased proliferation and apoptosis have been reported at microregions of extracellular matrix remodelling, suggesting a potential association between dysregulation of tenocyte homeostasis and changes in tendon extracellular matrix.³² A significant increase of apoptosis in human rotator cuff disease has been reported.^{23 29–31 33–35} However, the identification of apoptotic cells using TUNEL (terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick end labelling) in these studies is debatable as this method can generate false-positive results not discriminating necrotic, apoptotic and autolytic cell death.^{36–38} This makes the conclusion on the actual extent of tenocyte apoptosis in these studies uncertain. Furthermore, the relationship between tenocyte apoptosis and other features of tendon pathology remains unclear, as does the potential role of p53 – a key regulator of apoptosis and a potential regulator of tendon extracellular matrix homeostasis.

The objective of this study was to quantitate the rate of tenocyte apoptosis in torn supraspinatus tendons and in the matched subscapularis tendon, compared with a reference group of subscapularis tendons from patients with normal rotator cuffs. In addition, our aim was to determine the tenocyte density and proliferation rate in order to better understand the biological context of tenocyte apoptosis. Third, we assessed the potential role of p53 in torn rotator cuff, as this protein regulates many aspects of apoptosis in various pathological scenarios.³⁹

MATERIALS AND METHODS

Patients and tissue collection

The study was approved by the regional committee for research ethics. Informed consent was obtained. Patients were carefully selected; factors that could influence the occurrence of apoptosis such as nicotine use and systemic inflammatory disorders led to exclusion. Fifteen patients with supraspinatus tendon tear and no apparent pathology of the subscapularis tendon on MRI

and during arthroscopy were included. The cuff tears were repaired arthroscopically. Biopsies were harvested from the edge of the torn supraspinatus tendon and from the subscapularis tendon. The biopsy size was approximately 3×3×3 mm³. As a reference, samples of subscapularis tendon were collected from 10 patients undergoing arthroscopic labral repair with no apparent rotator cuff pathology. The subscapularis tendon samples were collected from the upper part of the tendon, approximately 1.5 cm from its insertion on the lesser tuberosity. For histology and immunohistochemistry, tissue samples were fixed in fresh 10% buffered formalin for 16–24 h at 4°C and then subsequently dehydrated and embedded in paraffin. For gene expression analysis, tissue samples from four rotator cuff tear patients and four patients of the control group were placed directly in RNA preservative (RNAlater; Ambion, Streetsville, Ontario, Canada) immediately after dissection, incubated at 4°C for 24 h and then stored at –20°C.

Tendon degeneration (tendinosis)

Sections of 5 µm were stained with H&E for morphology and with Alcian Blue for sulphated glycosaminoglycans (GAGs). Four diagnostic features of tendinosis – fibroblastic alterations (hyper/hypocellularity), increased GAG, collagen disorganisation or disarray and hypervascularity or vascular remodelling – were rated semiquantitatively. The extent of tendinosis was evaluated according to a modified Bonar scale by a single examiner (AS) who was blinded to the identity of the slides.⁴⁰ Using this scale, a completely normal tendon would score 0 and a maximally degenerated tendon would score 12. All subscapularis tendons, both from patients and controls, contained areas of chondrocyte-like cells; this may reflect zones of compression. Thus, this was considered a normal phenomenon and accordingly the evaluation of Alcian Blue staining was not included in the Bonar score. The validity of the Bonar scale in assessing tendon ruptures has been previously established.⁴¹

Tenocyte density

The identity of H&E-stained slides was masked with a dark tape. Light microscopy was performed on a digital imaging workstation (Veritas; Molecular Devices, Sunnyvale, California, USA) using a 20× objective lens (Zeiss, Toronto, Ontario, Canada). An automated scanning function was used to create a composite micrograph of the entire tissue section. Tenocyte density was determined by calculating the average for all individual fields that were free of significant artefact and contained only tenocytes. Cell counting was done independently by two observers (AS and KL). The procedure's reliability was evaluated by an interobserver test and was determined to be highly reproducible ($r^2=0.97$).

Detection of cellular proliferation

Reagents and antibodies for Ki67 immunohistochemistry were obtained from a single supplier (Ventana Medical Systems, Tucson, Arizona, USA). Staining was carried out using an automated immunohistochemistry unit (Discovery XT, Ventana, Tucson, AZ, USA) with an LSAB (labelled streptavidin-biotin, Ventana, Tucson, AZ, USA) kit according to the manufacturer's instructions, using a universal secondary antibody with 3,3'-diaminobenzidine as the substrate. Tissue sections were pretreated with EDTA buffer (CC1). The primary antibody was incubated at 37°C for 60 min and the secondary antibody at 37°C for 30 min. Human formalin-fixed paraffin-embedded tonsil was used for optimisation and as a positive control.

Detection of apoptosis

A monoclonal antibody against single-stranded DNA breaks (F7-26; Chemicon, Temecula, California, USA) was used to examine apoptotic cell death. Tissue sections were deparaffinised in xylene, then washed sequentially in 100%, 95% and 70% ethanol and phosphate-buffered saline (PBS). Slides were incubated in PBS containing 0.1% NP40 for 10 min followed by 20 µg/ml proteinase K for 40 min. Slides were washed in distilled water and transferred to 60°C, 50% formamide (v/v distilled water) for 20 min. Endogenous peroxidase activity was then blocked as above, followed by further blocking in goat serum for 20 min and in avidin D and biotin solutions for 15 min each. The sections were incubated with F7-26 (1:10 dilution) for 30 min at room temperature, then with goat antimouse immunoglobulin M (1:100; Dako, Mississauga, Ontario, Canada) for 15 min. Sections were then processed with Vectastain (Vector Labs, Burlingame, CA, USA) ABC reagents according to the manufacturer's instructions. Formaldehyde-fixed mammary rat tissue with or without the primary antibody was used as positive and negative controls.

Apoptotic index and proliferation index

The apoptotic index (AI) was determined for the F7-26 staining and the proliferation index for Ki67 staining. AI was defined as the percentage of apoptotic cells within all fields of a given biopsy that demonstrated positive labelling with the assay. The slide identity was masked with dark tape. Cell counting was done on a digital imaging workstation (Veritas; Molecular Devices), using 40× objective. Composite digital micrographs of the entire tissue section were generated as described above. In all fields containing positive cells, the number of both positive and negative cells was counted. The cell counting was done independently by two observers (AS and KL). The counting procedure's reliability was evaluated by an interobserver test and was determined to be highly reproducible ($r^2=0.964$).

p53

To further examine apoptotic cell death, we assessed the presence of p53 protein. p53 is a powerful tumour suppressor and promotes programmed cell death via apoptotic pathways.^{39 42} A monoclonal antibody (M7001; Dako) was used in conjunction with automated immunohistochemistry (Ventana) as described above. Heat-mediated antigen retrieval in EDTA buffer (CC1) was used. Primary antibody was incubated at 37°C for 60 min at 1:100 dilution, followed by secondary antibody at 37°C for 30 min. Human formalin-fixed breast cancer tissue was used for optimisation and as a positive control.

Data analyses

To compare the histological and immunohistochemical data between normal and tendinopathic tendons, the Mann-Whitney U test was conducted with p values less than 0.05 considered significant. Data were analysed using VassarStats online statistical software hosted by Vassar College (Poughkeepsie, NY). Reliability testing and correlation of variables within patients was conducted using Pearson's correlation coefficient.

Gene expression analysis

A subset of torn supraspinatus and reference subscapularis tendons were homogenised in a Mikrodismembrator (Sartorius, Germany) and the homogenate placed immediately in Trizol.

RNA was extracted and purified using RNEasy columns. cDNA was generated via reverse transcription (all reagents from Applied Biosystems). Gene expression analysis was performed using reverse transcriptase Profiler PCR Arrays (SABiosciences, Frederick, Maryland, USA) for p53-related genes. Data analysis was performed using SABiosciences Expression Analysis Software (v3.0; Frederick, MD, USA). Genes with both fold change >2.5 and $\alpha < 0.05$ were considered significant.

RESULTS

Patient characteristics

Fifteen patients, 10 men and 5 women, with full thickness tears of the supraspinatus tendon were included. The mean age was 57.7 years (range 49–69). The reference group consisted of 10 patients; mean age 43.9 years (range 32–51). They were five women and five men. No complications occurred.

Evaluation of tendon degeneration

Torn supraspinatus tendons consistently revealed significant degenerative change (ie, tendinosis). The main features were collagen disarray, areas of hypocellularity but more frequently areas of hypercellularity and signs of vascular proliferation or remodelling (neovascularisation). Fibroblastic cells predominated, and inflammatory cells were scarce or absent. Localised areas of increased tenocyte density were observed in the patient subscapularis tendons, but not the reference subscapularis tendons.

Blinded evaluation of the tissue samples using the modified Bonar scale confirmed the greater presence of degenerative change in the patient supraspinatus compared with reference subscapularis tendons and, interestingly, mild degenerative changes in the patient subscapularis tendons (figure 1A).

Apoptotic index

Apoptotic cells were distributed throughout the patient tendons, including supraspinatus and subscapularis tendons. There was a significant difference between the AI in torn supraspinatus and matched subscapularis compared with reference subscapularis tendons (patSSP (patient supraspinatus) vs reference subscapularis (refSSC) $p=0.019256$; patSSC (patient subscapularis) vs refSSC $p=0.041685$, figure 1B). Pearson's correlation coefficient confirmed a significant, positive correlation between the presence of apoptosis in torn supraspinatus and matched subscapularis ($R^2=0.5742$; $p=0.0005$) (figure 1C). This correlation indicates that apoptosis was not confined to

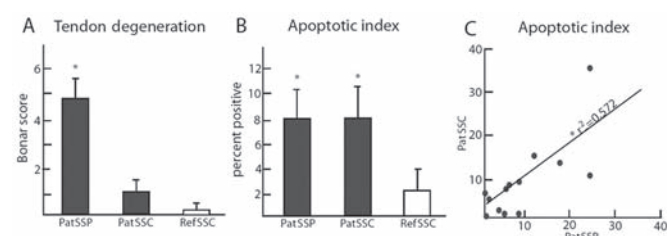


Figure 1 Lack of relationship between tendon degeneration and tenocyte apoptosis. (A) Bonar score demonstrated significant degeneration in patSSP tendon. (B) Apoptotic index was increased in both patSSP and patSSC tendons. (C) Apoptosis in patSSP and patSSC tendons were significantly correlated. PatSSC, patient subscapularis; patSSP, patient supraspinatus; refSSC, reference subscapularis. $*p < 0.05$.

the injured tendon, but also occurred to a similar degree in the matched, mildly degenerated but intact subscapularis tendon.

Tenocyte proliferation

The tenocyte density and proliferation indices in ruptured supraspinatus samples were significantly higher than the corresponding values for patient or reference subscapularis samples (figure 2A,B).

p53 protein and related gene expression

p53 immunohistochemistry showed a significantly greater expression in the torn supraspinatus than in the reference subscapularis ($p < 0.05$; figure 3A). In line with the immunohistochemical assay, p53 gene expression was 40% higher in injured supraspinatus tendon; however, this difference did not meet the criteria for statistical significance. Several genes encoding for p53 inhibitors were expressed at lower levels in patient tendons, including HDAC1 ($p < 0.05$), MDM4 ($p < 0.001$) and PPM1D ($p < 0.05$; figure 3B).^{43–45} In addition, the prosurvival nuclear transcription factor NF- κ B was downregulated ($p < 0.05$). Taken together, the results suggest that apoptosis in injured supraspinatus tendon is associated with multiple gene regulatory events relevant to p53 signaling and cell survival. However, p53 expression in the patient supraspinatus and subscapularis did not correlate with the

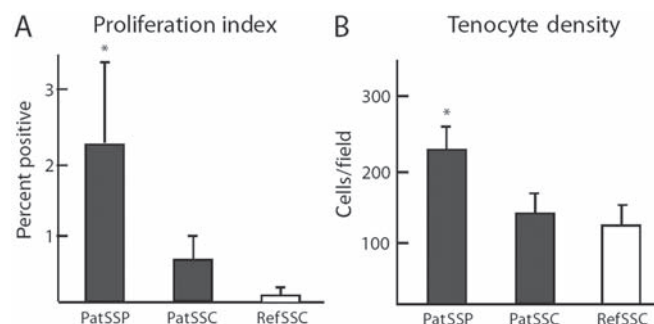


Figure 2 Tenocyte proliferation and tenocyte density in injured rotator cuff. (A) Proliferation index was determined by quantitating Ki67+ cells. (B) Cell density was determined by systematic counting of tenocytes on digital histological grids. PatSSC, patient subscapularis; patSSP, patient supraspinatus; refSSC, reference subscapularis. $*p < 0.05$.

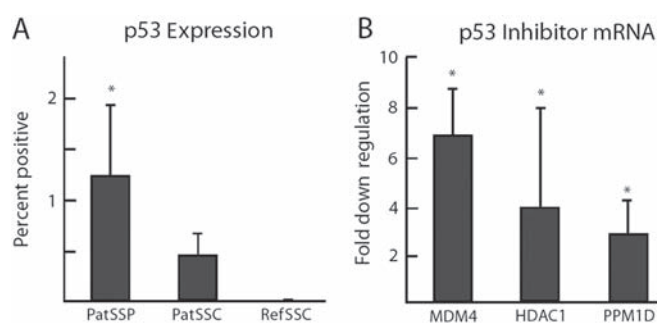


Figure 3 Potential roles of p53 in rotator cuff injury. (A) p53 expression was quantitated by counting of immunohistochemical specimen for patient and reference tendons. (B) Quantitative PCR (qPCR) was conducted to examine mRNA levels for genes related to p53 signalling in injured supraspinatus and reference control tendons. Error bars represent 95% CI. patSSC, patient subscapularis; patSSP, patient supraspinatus; refSSC, reference subscapularis. $*p < 0.05$.

AI in these tendons, indicating that p53 may be partially, but not solely, responsible for regulating tenocyte cell death in the injured rotator cuff.

DISCUSSION

The main goal of this study was to quantify the rate of tenocyte apoptosis in the torn rotator cuff of a well-defined population. We studied torn supraspinatus tendons and matched subscapularis tendons, comparing them with a reference group of subscapularis tendons from patients presenting with a normal rotator cuff. There was a significant increase of tenocyte apoptosis in both the torn supraspinatus tendon and the matched, intact subscapularis tendon. In addition, a significant increase in a key apoptotic regulator, p53, occurred specifically in the torn supraspinatus tendon and not in the matched subscapularis tendon, implying the existence of pathways both dependent and independent of p53 in the torn rotator cuff. Taken together, the findings demonstrate that tenocyte apoptosis is a more generalised feature of rotator cuff disease than previously appreciated, and that different apoptotic pathways may be activated in the torn supraspinatus compared with the adjacent uninjured cuff.

The extent of tenocyte apoptosis in our study is relatively small, but the magnitude of increase in the injured rotator cuff compared with healthy reference tendon (8.2% in torn supraspinatus tendon vs 2.8% in reference subscapularis tendon) is in keeping with the two- to threefold elevation reported in previously published studies.^{23 29–31} We excluded nicotine users since nicotine may promote apoptosis; patients with systemic disorders were also excluded. To our knowledge, this has not been done in previously published studies on apoptosis in rotator cuff tendinopathy. We identified apoptosis by studying the presence of ssDNA, a hallmark of apoptotic cell death.⁴⁶ The use of TUNEL in previous published studies is debatable as this method is criticised for generating false-positive results.^{36–38} Apart from the exclusion of nicotine users and patients with systemic disorders, this may be an additional explanation for the discrepancy between the AIs in our study and the studies from Yuan *et al*, Tuoheti *et al* and Benson *et al*.^{23 29 31} In addition, we only included apoptotic tenocytes/fibroblasts in our evaluation of the samples whereas Yuan *et al* and Millar *et al* did not seem to discriminate cell subpopulations in their evaluation.^{31 34}

Tendon healing after rupture is characterised by a brief inflammatory period, cell migration and proliferation phase and a prolonged remodelling phase. Increased apoptosis at the late stage of normal tendon healing *in vitro* has been identified as a possible mechanism to restore tissue homeostasis.¹⁵ Chuen *et al*⁶² described an increased rate of apoptosis in remodelling sites of healthy patellar tendons from active individuals. Understimulation of tenocytes following microscopic damage to tendon fibrils *in vitro* has been shown to result in apoptosis.⁴⁷ Wu *et al*¹⁷ documented the initial presence of increased apoptosis followed by proliferation in early avian tendon healing. Thus, several lines of evidence suggest that apoptosis could be triggered at different steps in the development of tendinopathy, from the accumulation of, and response to, micro-ruptures through to failure of the tendon. Our work extends these previous findings by showing that in patients with a torn rotator cuff, apoptosis also occurs in adjacent, macroscopically normal tendon.

Other work on patellar and rotator cuff tendinopathy has demonstrated the presence of increased tenocyte density and proliferation rate.^{25 48} These findings are confirmed and

extended by our study. Matthews *et al*⁴⁸ described increased cell density and proliferation in small and medium rotator cuff tears, compared with hypocellularity and reduced proliferation in large and massive tears. The tears were measured according to Post *et al*⁴⁹: small tears <1 cm, medium tears <3 cm and large tears <5 cm and massive tears >5 cm. All tears included in our study are medium tears. The potential relationship between tenocyte proliferation and early tendinosis changes has recently been demonstrated in a laboratory study of rotator cuff overuse.⁵⁰

One limitation of our study is the age difference between the two groups of patients: 57.7 years versus 43.9 years in the reference group. In previously published studies on apoptosis, this discrepancy is more pronounced with the exception of the study by Tuoheti *et al*,²⁹ which used autopsy controls. We find the age discrepancy in our material to be acceptable since degenerative changes of the rotator cuff are not appreciable until the fourth decade, thus placing both our groups at an age prone to tendinopathic change. This reduces the possibility of ageing alone as a cause of difference in AI. Another limitation of our study lies in the differences in anatomy, function and loading profile of the subscapularis tendon compared with the supraspinatus tendon. We consider biopsying healthy supraspinatus tendon from the living to be unethical because of the known reduced healing potential of this tendon. MRI was performed at different institutes and this clearly is a possible limitation allowing variance in the radiologic evaluation of the subscapularis tendon. Any visible pathology of the subscapularis tendon during arthroscopy led to exclusion from the study independent of the MRI result. A general limitation of human studies on tendinopathy is the fact that tendon samples represent tissue with advanced disease not enabling observation of early pathological features. A final limitation is that the extent of involvement and cellular changes in the rotator cuff muscles prior to or following tendon failure are not known. These are important issues that require further investigations. The involvement of the subscapularis tendon in the presence of a supraspinatus tear in our study confirms the findings of Yuan *et al* in 2002³¹ and reinforces the importance of this tendon in the study of rotator cuff tendinopathy.

This study indicates a general involvement of the rotator cuff in the presence of a supraspinatus tear, and a potential role of both p53-dependent and -independent cell death. Apoptosis is an observable feature of tendinopathy, however,

What is already known on this topic

Recent research has indicated that apoptosis plays a role in rotator cuff tendinopathy. The actual extent of tenocyte apoptosis in the torn supraspinatus, the matched intact subscapularis, and normal rotator cuff is unclear.

What this study adds

This study indicates a general involvement of the rotator cuff in the presence of a supraspinatus tear, and a potential role of both p53-dependent and -independent cell death. Apoptosis is an observable feature of tendinopathy however its contribution to tendon degeneration requires further research.

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Competing interests None.

Patient consent Obtained.

Ethics approval This study was conducted with the approval of the Lovisenberg Deaconal Hospital.

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