Cytokines and apoptosis in supraspinatus tendinopathy

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The role of inflammatory cells and their products in tendinopathy is not completely understood. Pro-inflammatory cytokines are upregulated after oxidative and other forms of stress. Based on observations that increased cytokine expression has been demonstrated in cyclically-loaded tendon cells we hypothesised that because of their role in oxidative stress and apoptosis, pro-inflammatory cytokines may be present in rodent and human models of tendinopathy. A rat supraspinatus tendinopathy model produced by running overuse was investigated at the genetic level by custom micro-arrays. Additionally, samples of torn supraspinatus tendon and matched intact subscapularis tendon were collected from patients undergoing arthroscopic shoulder surgery for rotator-cuff tears and control samples of subscapularis tendon from ten patients with normal rotator cuffs undergoing arthroscopic stabilisation of the shoulder were also obtained. These were all evaluated using semiquantitative reverse transcription polymerase chain-reaction and immunohistochemistry.

We identified significant upregulation of pro-inflammatory cytokines and apoptotic genes in the rodent model (p = 0.005). We further confirmed significantly increased levels of cytokine and apoptotic genes in human supraspinatus and subscapularis tendon harvested from patients with rotator cuff tears (p = 0.0008).

These findings suggest that pro-inflammatory cytokines may play a role in tendinopathy and may provide a target for preventing tendinopathies.

Tears of the rotator cuff are a cause of considerable pain and dysfunction in the shoulder in adults. The results of surgical repair of full-thickness tears have been favourable, but post-operative imaging has shown high rates of re-tearing indicating failure of the biological healing of the tendon-bone interface. The subacromial bursa and the torn margin of the rotator cuff have been suggested as potential sources of healing with studies implicating the role of cytokines, matrix metalloproteineases, oxygen-free radicals and growth factors in the pathophysiology of rotator-cuff tears.

Cytokines are important molecular messengers in the response of soft tissue to injury and wound healing. They have been shown to be involved in the regulation of matrix turnover in tendinopathy and may have a direct effect on the activity of tenocytes and the subsequent expression of tendon matrix genes. Cytokines also play a key role in oxidative stress-induced cellular apoptosis which is mediated by the activation of caspases (cysteine-containing aspartic proteases, a group of proteolytic enzymes) and is involved in the stress-induced cascade of tendinopathy.

Based on our previous observations of increased apoptosis in tendinopathy we hypothesised that because of their central role in caspase-dependent apoptosis, pro-inflammatory cytokines may be present in human and rodent models of tendinopathy.

Materials and Methods
We randomised equally 24 male, 30-week-old Sprague-Dawley rats weighing a mean of 362 g (342 to 388) into an exercise and control group. The exercise group underwent a daily treadmill running regimen to model tendon overuse resulting in degeneration as previously described. The rats initially underwent daily training which increased in duration over two weeks to accustom them to the exercise and surroundings. After this, they were subjected to exercise which consisted of running on a 10° decline at 17 m/min for one hour per day, five days per week. This regimen equates approximately to 7500 strides/day. After running for four weeks the rats were killed by CO₂
inhalation and both supraspinatus tendons were collected. The control group consisted of 12 rats which did not have exercise. All the procedures and protocols were approved by the Animal Care and Ethics Committee of the University of New South Wales under ACEC No. 99/101.

In addition we obtained 17 specimens of supraspinatus tendon from patients with tears of the rotator cuff undergoing surgery (Table I). Their mean age was 57 years (39 to 75) and the mean size of tear was 3.2 cm² (1 to 45). The operation was performed using a standard arthroscopic technique. The size of the tear was recorded at the time of the operation. Clinical examination included Hawkins impingement sign5 and strength testing of the supraspinatus using a handheld HFG-45 force gauge (Transducer Techniques, Temecula, California) as previously described by Hayes et al.16 Samples of the subscapularis tendon were also collected from the same patients. They were only included if there was no evidence of subscapularis tendinopathy on a pre-operative MR scan or of macroscopic damage to the tendon at the time of arthroscopy.

As an independent control group ten samples of subscapularis tendon were collected from patients undergoing arthroscopic surgery for shoulder stabilisation who did not have tears of the rotator-cuff confirmed by arthroscopic examination. The mean age of the control group was 35 years (20 to 41).

The subscapularis tendons were harvested arthroscopically from the superior border of the tendon 1 cm lateral to the glenoid labrum and the supraspinatus tendons from within 1.5 cm of the edge of the tear before surgical repair. All the specimens were immediately placed in RNAlater solution (Ambion Inc, Austin, Texas) and stored at -20°C until RNA extraction. For immunohistochemical staining the tissue samples were immediately fixed in 10% (v/v) formalin for four to six hours and then embedded in paraffin. Custom micro-arrays consisting of 5760 rat oligonucleotide features in duplicate were provided by the Ramaciotti Centre of the University of New South Wales, Sydney, Australia. A total of 15 μg to 20 μg of total RNA was used to synthesise complimentary DNA (cDNA) in which Cy3 or Cy5 fluorescent dyes (Amersham Pharmacia Biotech, North Ryde, Australia) were incorporated using an indirect labelling method. Micro-arrays were scanned using an Axon GenePix micro-array scanner (Molecular Devices, Union City, California) and the resulting image analysed using GenePix prosoftware version 3.0 (Molecular Devices). All the genes were represented by at least two independent targets on each micro-array so that at least six independent targets were identified across three micro-arrays. The signal from each target was used to calculate a mean signal for each gene. The latter was considered to be up- or down-regulated if it had at least a 1.5-fold difference from the control group.

Total RNA was isolated from tendon tissue using Trizol reagent (Life Technologies, Grand Island, New York) according to the manufacturer’s instructions. The RNA yield and integrity were evaluated by spectrophotometry and agarose gel electrophoresis, respectively. The optical density 260/280 ratio was maintained between 1.7 and 2.1, confirming that the quality of the RNA was satisfactory.

RNA extracted from tendon tissue was reverse transcribed to generate the first strand of cDNA and subsequently amplified using a polymerase chain-reaction (PCR) to produce amplified targeted segments of cDNA using the ImProm-II Reverse Transcription System.

Table I. Details of the 17 patients

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<th>Age (yrs)</th>
<th>Duration of symptoms (mths)</th>
<th>History of traumatic rotator cuff injury</th>
<th>Pain intensity score¹</th>
<th>Pain frequency score¹</th>
<th>Supraspinatus strength (N)</th>
<th>Impingement sign²</th>
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* the pain intensity occurring at night, at rest, and with activities was scored as follows: 0, no pain; 1, mild pain; 2, moderate pain; 3, severe pain and 4, very severe pain, and the three scores combined (maximum 12)
† the frequency of severe pain, pain at night, and pain with activities was scored as 0, never; 1, monthly; 2, weekly; 3, daily; and 4, constantly and the three scores combined (maximum 12)
‡ Hawkins impingement sign
(Promega, Madison, Wisconsin). All the reverse transcription steps were performed in a GeneAmp 2400 thermal cycler (Applied Biosystems, Foster City, California). We mixed 1 μg to 5 μg of total RNA with 0.5 μg of oligo (dT) nucleotides primer to make a 5 μl solution and denatured it at 70°C for five minutes. The reaction mixture was then cooled to 4°C for at least five minutes. Subsequently, a 15 μl reverse transcription reaction mixture consisting of 1 μl of ImProm-II Reverse Transcriptase, 0.5 mM d nucl eoside triphosphates dNTPs, 6m M MgCl2, 4 μl of ImProm-II 5× reaction buffer, 20 U of Recombinant RNasin ribonuclease inhibitor and nuclease-free water, was added to the target RNA and primer combination. The reaction mix was heated to 70°C for five minutes to allow annealing of the oligo dT primers to the RNA. It was then incubated at 37°C for one hour in order to create the cDNA. For each tested RNA sample, a control reaction was run with Moloney murine leukemia virus (MMLV) reverse transcriptase omitted from the mixture to exclude false-positive results from genomic DNA contamination.

We amplified 1 μl (100 ng) of each cDNA sample from the above extractions by PCR (GeneAmp System 2400) in a total volume of 20 μl. Platinum Super Mix (22 U/ml of complexed recombinant Taq Polymerase with platinum Taq antibody, 22 mH Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM MgCl2, 220 μM dGTP, 220 μM dATP, 220 μM dTTP, 200 μM cCTP, stabilisers; Invitrogen Life Technologies, Carlsbad, California) was used together with 0.2 μM each of the specific oligonucleotide primers using published sequences (Table II). The thermal cycling programme consisted of an initial denaturation step of 95°C for 1 min 15 s, followed by 35 cycles of 95°C for 30 s, a 30-second annealing step at varying temperatures and a 30-second extension step at 73°C. Reaction products (12 μl) were analysed by electrophoresis through 3% agarose gels stained with ethidium bromide and visualised by transillumination under ultraviolet light at a wavelength of 302 nM. Amplification of the mRNA of β-actin was used as an internal control in all cases.

Paraffin sections were de-waxed with xylene and graded ethanol. Sections were stained with haematoxylin and eosin and Toluidine Blue. The presence or absence of oedema and degeneration was recorded, together with the degree of fibroblast cellularity, amyloid deposition and chondroid metaplasia. Tendinopathy was assessed on a basic histological scale derived from Khan et al17 (grade 4, marked degeneration; 3, advanced degeneration; 2, mild/moderate degeneration; 1, normal tendon) by two independent assessors (AQW, FB) who were blinded to the results.

For immunohistochemical techniques antigen retrieval was achieved using Dako target retrieval solution (Dako, Carpinteria, California) in accordance with the manufacturer’s instructions. Endogenous peroxidase activity was scavenged with 3% (v/v) H2O2 and non-specific antibody binding blocked with 10% (w/v) milk in Tris-buffered saline (TBS) buffer for 15 minutes. Tissue slides were incubated with primary antibody (Interleukin (IL)-12, IL-15, IL-18, macrophage inhibitory factor (MIF) and tumour necrosis factor alpha (TNF-α)) diluted 1:100 in 1% (w/v) bovine serum albumin (BSA)/TBS at room temperature for 60 minutes. After two washes, the slides were incubated with Dako LINK consisting of biotinylated anti-mouse and anti-rabbit immunoglobulin (Ig). They were washed and incubated with streptavidin-peroxidase, followed by extensive washing and exposure to Dako liquid

<table>
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<th>Product</th>
<th>Primer sequence</th>
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<td>Caspase 8</td>
<td>Forward ACA GTG AAG TCT GGC CTC C, Reverse GCA GTG TCA TGT CAT CAT CC</td>
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<td>TNF-α</td>
<td>Forward AAG AGT TCC CCA GGG ACC CCT TCT, Reverse CCT GGG AGT AGA TGA GGT ACA</td>
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* bp, base pairs
† MIF, macrophage inhibitory factor
diaminsbenzidine (DAB) for five minutes. Finally, the sections were counterstained with haematoxylin. Positive and negative control specimens were included, in addition to the surgical specimens for each individual antibody staining technique.

**Statistical analysis.** The results were reported as the mean and SEM. Comparisons between groups were made using a two-way paired Student’s t-test, the Mann-Whitney U test and Kruskal-Wallis one-way analysis of variance on ranks using Sigma Stat version 3.1 (Systat Software Inc., Richmond, California). Corrections between groups used the Pearson’s correlation coefficient. A power analysis was performed with the beta error set at 0.2 (power = 0.8). Based on the results of this, it was determined that each group required ten tissue samples to detect a difference of 20% between each of the groups with regard to the gene expression. Statistical significance was set at a p-value ≤ 0.05.

**Results**

**Rat micro-array.** Of the 5760 independent targets on each of the micro-arrays, most had less than a twofold change in expression in the experimental samples compared with the control group and thus were considered not to be differentially expressed. Overall, 91 genes were found to be significantly upregulated and 37 significantly downregulated as previously reported by Molloy et al18 from our laboratory. Inflammation-related genes were also over-represented in degenerated tendon, with 14 genes (15.4%) significantly upregulated and five (13.5%) significantly downregulated. Upregulation (p < 0.01, Student’s t-test) of IL-11 (× 3.6), IL-15 (× 2.5) and IL-18 (× 1.8), anti-NGF30 (× 2.6), MIF (× 1.8), globin transcription factor (GATA) binding protein 3 (× 3.3), platelet-activating factor acetylhydrolase (× 3.1), CD3 gamma chain (× 3.1), IgG-2b gene (× 3.0), T-cell receptor variable β chain (× 4.6), IL-6 (× 2.3), attractin (× 2.4), TNF-α receptor (× 2.5) and receptor activator of NF-kb ligand (× 3.0) and downregulation (p < 0.05, Student’s t-test) of Ig heavy chain (× 3.0), CD94 (× 2.8), T cell receptor α chain (× 6.5), IL-2 (× 3.3) and T-cell receptor (× 2.2) occurred in degenerative rat supraspinatus tendon subjected to daily treadmill running for four weeks. The differential expression of apoptotic-related genes was represented by five upregulated genes (5.5%) and three downregulated genes (8.1%). Upregulation of heat shock protein 27 (× 3.4 and 70 (× 2.5), cellular FLICE inability protein (cFLIP) receptor (× 2.2) and caspase 8 (× 3.1) while downregulation of poly(ADP-ribose) polymerase (× 4.8), type-2 angiotensin II receptor (× 2.6) and hypoxia-inducible factor 1 (× 2.2) were noted.

Also differentially expressed were cellular communication genes, including members of the glutamate signalling machinery, represented by 12 significantly upregulated genes (13.2%) and three significantly downregulated genes (8.1%). Several growth, differentiation, and developmental genes were also differentially regulated in the degenerated tendons, represented by 13 upregulated genes (14.3%) and ten downregulated genes (11%).

**Human tendon samples.** The cytokine genes IL-18, IL-15, IL-6, MIF and TNF-α were detected in all samples of torn supraspinatus tendon. The expression levels of IL-18, IL-15, IL-6 and MIF was higher (p < 0.001, Kruskal-Wallis ANOVA) in the torn edges of supraspinatus when compared with matched subscapularis tendon (Fig. 1). The expression of the apoptotic genes caspases 3 and 8 were among the most significantly increased in the same tissue samples (p = 0.0005). TNF-α mRNA expression was elevated (p < 0.05, Student’s t-test) in subscapularis tendon compared with torn supraspinatus samples. The levels of expression of IL-18, IL-15, IL-6, MIF, TNF-α and caspases 3 and 8 were elevated (p < 0.001, Kruskal-Wallis ANOVA) in torn supraspinatus and matched subscapularis when compared with normal control subscapularis. We observed no correlation between tear size (r = 0.1, p = 0.6), patient age (r = 0.2, p = 0.8), pain (r = 0.1, p = 0.9) and cytokine (r = 0.4, p = 0.8) and apoptotic (r = 0.3, p = 0.5) gene expression. These apoptotic and cytokine genes were confirmed at the protein level at which IL-18, IL-15, IL-6, MIF, TNF-α, and caspases 3 and 8 were confirmed in all samples of torn supraspinatus tendon (Fig. 2). Increased (p < 0.001, Kruskal-Wallis ANOVA) immunoactivity of IL-18, IL-15, IL-6, MIF and caspases 3 and 8 were found in torn supraspinatus compared with matched and normal subscapularis. The proteins were localised to tendon cells. Negative controls using human epidermis showed no expression of any apoptotic genes. Positive controls of human tonsil tissue confirmed the presence of IL-18, IL-6, MIF, TNF-α and caspases 3 and 8. IL-15 was identified in human liver tissue as a positive control.

Of additional interest haematoxylin and eosin staining of matched subscapularis samples showed overall appearances of advanced degenerative change consistent with tendinopathy with seven samples showing grade-3 and nine grade-2 changes despite no evidence of tendinopathy on pre-operative MRI and macroscopically at the time of arthroscopy (Fig. 3). All torn supraspinatus tendons showed features in...
keeping with those of advanced mucoid change and chondroid metaplasia as expected in association with marked degenerative change consistent with tendinopathy (grade 4). In a previous investigation matched subscapularis tendons from patients with full-thickness tears of the rotator cuff had approximately 35% of apoptotic cells compared with 20% in the subscapularis tendon from patients without a tear confirming our histological finding of a degenerative process in supposedly normal samples of subscapularis tendon. We observed no correlation between tear size (r = 0.1, p = 0.6), patient age (r = 0.2, p = 0.5), pain (r = 0.1, p = 0.6) and the histological grade of tendinopathy (r = 0.8, p = 0.2) Pearson’s correlation coefficient.

Discussion
We wished to investigate further the role played by cytokines in the stress-induced apoptosis pathway of tendinopathy. Our data were the first to confirm the presence of the cytokines IL-18, IL-15 and IL-6 and MIF in both rat and human models of tendinopathy. We also identified significantly increased levels of key mediators, caspases 3 and 8 and of Fas-Ligand apoptosis in the margins of the rotator cuff of patients with full-thickness tears compared with matched subscapularis tendon and normal independent control tendon.

Cytokines have been shown to be important contributors to the pathology of tendinopathies. In animal studies local administration of cytokines induced a histological
picture of tendinopathy while application of cyclical strain increased the presence of IL-6 and 1β gene expression in human flexor tenocytes. Lin et al have recently shown that IL-6 knockout mice show inferior mechanical and organisational tendon properties compared with a control group, while Nakama et al highlighted the role of IL-6 in stimulating transcription factors in human rotator-cuff tendon. Others investigating the therapeutic benefit of blocking cytokines have shown that in doing so collagenase concentrations and angiogenesis can be reduced in rheumatoid tenosynovium which ultimately reduces tendon damage.

Cytokines also play a key role in oxidative-stress-induced cellular apoptosis. Several groups have highlighted the role of apoptosis in degeneration of tendon cells with excessive apoptosis noted in the torn edges of rotator-cuff tendons, and in a mechanically stained tibialis anterior rodent model. Apoptotic cell death is orchestrated by the activation of caspases, a family of cysteine proteases for aspartic-acid residues, which cleave specific intracellular substrates to produce the characteristic features of this form of cell death. Previous work has shown that oxidative-stress-induced apoptosis in human tendon fibroblasts is mediated by the release of the cytochrome C pathway and activation of caspase 3. Recently, we discovered increased levels of heat shock proteins in human rotator cuff. These are known to protect cells from the cytotoxic effects of cytokines and apoptotic mediators. Another group has discovered a genetic component to rotator-cuff tears which may also be linked to the apoptotic process.

In both our models of rat and human tendinopathy increased levels of cytokines involved in oxidative-stress-induced apoptosis were identified as was the expression of key apoptotic regulatory genes. MIF has been shown to have pro-inflammatory effects in a wide range of diseases including atherosclerosis and rheumatoid arthritis. MIF induces the gene expression of other inflammatory cytokines (TNF-α, IL-6), while overexpression suppresses pro-oxidative stress-induced apoptosis. IL-18 increases the synthesis of nitric oxide and induces apoptosis in human articular chondrocytes and bone-marrow cells. IL-15 is a potent inhibitor of several apoptotic pathways including ligand-induced association of TNR receptor 1. TNF-α is a classic initiator of Fas ligand and caspase-8-derived apoptosis and plays a role in chondrocyte apoptosis while IL-6 has been shown to have several anti-apoptotic effects.

Based on our observations the overexpression of these cytokines in these models of tendinopathy alongside increased apoptotic genes suggests that these molecules play key effector roles in the Fas-Ligand pathways in an attempt to regulate the degree of apoptosis. They may act to up- or down-regulate apoptosis and as such may be either protective or harmful to tendon cells.

Oxygen-free radicals and stress-activated protein kinases have also been implicated in the stress-induced apoptotic cell pathway. IL-18, IL-15 and IL-6 are known to promote the intensive production of reactive O2 species and protein kinases. Our finding of significantly increased cytokine levels may suggest that these molecules when expressed during the degenerate and healing phases of tendon injury, result in the subsequent production of reaction O2 species and protein kinases causing tendon damage or failure of the normal reparative process.

We found no correlation between size of tear, age, patient pain scores, or the duration of symptoms with the histological grade and gene expression in all samples. Other groups have shown a correlation between tear size and the degree of cellular metabolism and cellular and gene expression in all samples. Other groups have shown a correlation between tear size and the degree of cellular metabolism and cellular and gene expression in all samples. Other groups have shown a correlation between tear size and the degree of cellular metabolism and cellular and gene expression in all samples.
Ultimately there remains a complex regulatory network between inflammatory cytokines and apoptosis in tendon cells. It may be that apoptotic cell death induced by cytokines in tissues exposed to normal stresses is a routine process used to remove partially damaged cells while excessive stresses may cause increased production of cytokine leading to increased apoptosis and ultimate cell necrosis. We therefore hypothesise, based on our findings and those of others, that an increase in the amount and duration of load in a tendon cell may result in the activation of cytokines, stress-activated protein kinases, oxygen-free radicals and apoptotic mediators which when persistently activated cause the tendon cells to undergo apoptosis resulting in a weakened collagenous matrix leading to subsequent tendon degeneration and clinical tendon rupture.

There are limitations to our study. First, age-related changes within the tendon samples could contribute to the overall degenerative picture and gene expression seen in the matched subscapularis tendons. However, the lack of degenerative change on MRI and arthroscopic examinations suggests that the differences are truly at the cellular level as suggested by our work. Secondly, the rodent model of tendinopathy is unlikely to represent human age-related degenerative changes and thus would not reflect this in the microarray data. Thirdly, subscapularis tendon is functionally and organisationally distinct from supraspinatus and thus responds to mechanical loading in a different manner which may alter its cytokine gene profile. Its genetic profile may also be altered from cytokines released from supraspinatus into the glenohumeral joint. It is reassuring, however, that we also be altered from cytokines released from supraspinatus which are being tested clinically in other inflammatory conditions with promising results. Our finding of an increase in inflammatory cytokines in early and late tendinopathy highlights their role in the degenerative process and may provide novel targets in reducing the degree of tendon damage.

CytoKines and Apoptosis in Supraspinatus Tendinopathy

No benefits in any form have been received or will be received from a commercial party related directly or indirectly to the subject of this article.

References


Supplementary material

A further opinion by Dr P. Lapner is available with the electronic version of this article on our website at www.jbjs.org.uk


