Is resection of the tendon edge necessary to enhance the healing process? An evaluation of the homeostasis of apoptotic and inflammatory processes in the distal 1 cm of a torn supraspinatus tendon: part I

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**Background:** We hypothesize that the expression of proapoptotic and antiapoptotic molecules and cytokines is dependent on the distance from the torn supraspinatus tendon edge and this expression may influence its potential for healing. The aim of this work is to evaluate the expression of proapoptotic Bax molecule and caspases 3, 8, and 9; antiapoptotic Bcl-2 molecule; and proinflammatory tumor necrosis factor (TNF-α) and anti-inflammatory interleukin 10 (IL-10) in 3 sections taken from a 1-cm section of the edge of a torn supraspinatus tendon: 3 mm distal and 3 mm proximal, as well as the remaining 4-mm middle section between them.

**Methods:** Nine patients, with a mean age of 58 years, were included in the study. All fulfilled strict inclusion criteria regarding the morphology of the tear and reconstruction technique. Samples were taken from the ruptured supraspinatus tendon at the time of arthroscopic repair. Quantitative real-time polymerase chain reaction assay was used for analysis.

**Results:** The expression of caspases 9, 8 and 3; Bax; and TNF-α significantly decreased from the distal to the proximal parts of the tendon edge (P < .05). However, a significant increase in Bcl-2 and IL-10 expression was also found in the same direction (P < .05).

**Conclusions:** Tenocytes can reduce the expression of proapoptotic caspases 3, 8, and 9 and Bax, as well as proinflammatory TNF-α, by increasing the expression of Bcl-2 and IL-10 within 1 cm of the supraspinatus edge in a distal to proximal direction. Resection 4 to 7 mm from the edge of the torn supraspinatus tendon may enhance the healing process by reaching a reasonable compromise between molecular homeostasis of apoptotic and inflammatory processes and mechanical aspects of rotator cuff reconstruction.
Despite decades of experience in tackling the problem, the reconstruction of a damaged rotator cuff often results in the tear recurring.4,5,16,21,35,42 The recurrence of the tear leads to the progression of degenerative changes in the muscle, decreasing its strength further.12 It has been shown experimentally that, in the case of second-degree fatty degeneration of the supraspinatus or higher, according to the Goutallier computed tomography classification, considerable morphometric alterations occur.10,11 Despite this, the muscle is still able to generate 34% of control-value strength.11 Hence, it is vital for the reconstructed tendon to heal completely.12

A review of the current literature indicates that one reason for the healing difficulties seen to affect the repaired tendon may be apoptosis,2,22,24,25,27,28,40,41 as well as upregulation of the proinflammatory cytokines associated with the homeostasis of its extracellular matrix.27,40 Although earlier research has shown a greater degree of apoptosis2,22,24,25,27,28,40,41 and a higher concentration of proinflammatory cytokines to be present in the torn supraspinatus tendon27 and the synovium,34 there are no data regarding the defense activity of the tenocytes in response to increased concentrations of antiapoptotic molecules and anti-inflammatory cytokines. A greater understanding of the homeostatic basis of the apoptotic and inflammatory processes should enable a more comprehensive estimation of the healing potential of the torn tendon edge and better establish the degree of resection needed to increase the chance of healing.

The hypothesis of the study is that the expression of proapoptotic and antiapoptotic molecules, as well as proinflammatory and anti-inflammatory cytokines, depends on the distance from the edge of the torn supraspinatus tendon and that this trend may determine the potential for the rotator cuff to heal at any one point. The aim of this work is to evaluate the expression of proapoptotic factors (Bax proteins and caspas 3, 8, and 9) antiapoptotic factors (Bcl-2 proteins), as well as proinflammatory cytokine tumor necrosis factor (TNF) α and anti-inflammatory interleukin (IL) 10, in 3 sections taken from a 1-cm section of the resected margin of a torn supraspinatus tendon: a 3-mm section from the distal end, a 3-mm section from the proximal end, and the remaining 4-mm middle section.

Materials and methods

Informed written consent was obtained from all patients. The study included 9 patients: 7 men and 2 women. The mean age of the patients was 58 years (range, 51-66.1 years; SD, 5.01 years). Samples were taken from the superior part of the ruptured supraspinatus tendon at the time of arthroscopic repair of a U-shaped rotator cuff tear.7 The inclusion criteria were 3-fold: (1) the tear dimension was between 2 cm and 3 cm from lateral to medial and between 2 cm and 2.5 cm from anterior to posterior; (2) the tear extended through the full thickness of the supraspinatus and the anterior part of the infraspinatus,20 with an intact subscapularis tendon; and (3) repair using the margin-convergence technique was successful,5 with acceptable tension after a 1-cm-long and 1-cm-deep resection of the superior edge of the supraspinatus tendon (cuff mobility was evaluated through stitches after prior mobilization and selective capsulotomy, cutting the coracohumeral ligament from the coracoid process).

The criteria for exclusion were as follows: the absence of concomitant disorders such as biceps pathology requiring tenodesis or tenotomy, fractures, rheumatoid arthritis, osteonecrosis, glenohumeral arthritis, or labral pathology; the patient was receiving steroid injections; the patient was not in good general condition; and the patient was a smoker.

All samples were placed in Trizol reagent (Ambion, Foster City, CA, USA). Before examination, the edges of the samples were cut off and the specimens cut into 1 × 1-cm squares. Next, 3 mm of material was cut from the tear-margin end of the sample (distal), 3 mm was cut from the proximal end, and the remaining 4 mm formed a middle section. Each section was divided into 3 sections to give 3 measurements from each part, which were then combined to give an overall mean value for the section. The expression of caspases 9, 8, and 3 and the proapoptotic Bax and antiapoptotic Bcl-2 molecules, as well as TNF-α and IL-10 cytokines, was assessed according to Gach et al.13

Quantitative real-time polymerase chain reaction assay

All molecular assays were performed as previously described. The expression of human Bax; Bcl-1; TNF-α; IL-10; glyceraldehyde 3-phosphate dehydrogenase (GAPDH); and caspases 3, 8, and 9 was quantified by real-time polymerase chain reaction (PCR) using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer’s protocol. Total cellular RNA (1 μg) from the studied material was extracted by use of Trizol reagent (Invitrogen [Thermo Fisher Scientific], Carlsbad, CA, USA) using a single-step purification protocol.3 RNA pellets were dissolved in ribonuclease-free water, and their concentrations and purity were determined by spectrophotometer readings at 260 and 280 nm. RNA that had undergone polyadenylation was isolated by use of an Oligotex kit (Qiagen, Chatsworth, CA, USA): 50 ng of poly(A) RNA was used for the first-strand complementary deoxyribonucleic acid (cDNA) synthesis with the SuperScript II ribonuclease Transcriptase System (Invitrogen), using Oligo(dT)12-18 Primers (Invitrogen) as described per the manufacturer’s instructions. We amplified cDNA
with specific primers for messenger ribonucleic acid (mRNA) of human Bax; Bcl-1; TNF-α; IL-10; and caspases 3, 8, and 9. In the same samples, GAPDH mRNA was amplified with specific primers and used as an active and endogenous reference to correct for differences in the amount of total RNA added to the reaction mixture and to compensate for different levels of inhibition during reverse transcription of RNA and during PCR. In brief, 2.5-, 2.0-, 1.5-, 1.0-, 0.5-, and 0.25-μL samples of synthesized cDNA were amplified in triplicate for both GAPDH and each of the target genes to create a standard curve. Likewise, 2 μL of cDNA was amplified in triplicate in all isolated samples for each primer-probe combination and GAPDH. Each sample was supplemented with both 0.3-μmol/L forward and reverse primers, as well as a fluorescent probe, and was made up to 50 μL using qPCR Mastermix for SYBR Green I (Eurogentec, Seraing, Belgium). All PCR primers were designed using PrimerExpress software (Applied Biosystems).

Each target probe was amplified in a separate 96-well plate. All samples were incubated at 50°C for 2 minutes and at 95°C for 10 minutes and then cycled at 95°C for 30 seconds, 56°C for 1 minute, and 72°C for 1 minute for 40 cycles. An ABI Prism 7000 system (Applied Biosystems) was used to collect SYBR Green I fluorescence emission data, and mRNA levels were quantified using the critical threshold (C \text{t}) value. Controls without reverse transcription (RT) and with no template cDNA were included in each assay. To compensate for variations in input RNA amounts and reverse transcription efficiency, GAPDH mRNA was quantified and the results were normalized to these values. Relative gene expression levels were obtained using the ΔΔC \text{t} method of Winer et al. The results were presented as mean ± standard deviation.

Statistical analysis

The arithmetic mean and standard deviation were calculated from the basic position measurements. The Shapiro-Wilk test was used to check whether the values were normally distributed. A Wilcoxon nonparametric rank sum test was used with \( P < .05 \) considered significant. All calculations were performed by use of Statistica, version 10 (StatSoft, Tulsa, PL).

Results

Measurements of caspase expression in the distal, middle, and proximal parts of the resected edge of the supraspinatus tendon showed decreased expression of caspases 9, 8, and 3; Bax molecule; and TNF-α. However, the Bcl-2 and IL-10 expression was found to increase from the distal part to the proximal part of the resected tendon edge (Table I). The statistical analysis showed a significant \( (P < .05) \) decrease in the expression of caspases 9 and 3 and the proapoptotic Bax molecule between the distal and middle parts, distal and proximal parts, and middle and proximal parts of the tendon edge. A statistically significant decrease in caspase 8 expression was detected between the distal and proximal parts of the supraspinatus samples, as well as between the middle and proximal parts. The expression of antiapoptotic molecule Bcl-2 3 showed an opposite trend—a statistically significant increase from distal to proximal \( (P < .05) \). The level of expression of TNF-α significantly decreased and that of IL-10 significantly increased \( (P < .05) \) from distal to proximal between the distal and middle parts, distal and proximal parts, and middle and proximal parts (Table II).

The expression of the key apoptosis activator caspase 9 was much higher than that of molecule Bcl-2 in the proximal part of the supraspinatus tendon edge, whereas higher levels of the antiapoptotic molecule Bcl-2 were seen in the middle and proximal parts \( (P < .05) \) (Table III). Regarding the cytokines, although the expression of TNF-α was greater than that of IL-10 in the distal part, the opposite was true in the proximal part of the supraspinatus tendon edge; balance was reached between the two cytokines in the middle part (Table III).

Discussion

Careful resection of the torn edge of the rotator cuff is recommended by the pioneer of shoulder surgery, Neer, as the simplest method to increase the chances of the tendon healing. However, the need for torn rotator cuff edge resection to support the healing process is still the subject of much controversy, and an understanding of the potential of human supraspinatus tenocytes to control molecular homeostasis of apoptotic and inflammatory processes may prove valuable in this respect.

This study shows, for the first time, that the expression of proapoptotic molecules (caspases 3, 8, and 9 and Bax) and the expression of the proinflammatory cytokine TNF-α decrease in a distal to proximal direction within 1 cm of the edge of a torn supraspinatus tendon. Conversely, it also shows that the expression of the antiapoptotic molecule Bcl-2 and the expression of the anti-inflammatory cytokine IL-10 increase in the same direction (Tables I and II).

Apoptosis is a physiological process regulating tenocyte homeostasis and influences the structure and function of the tendon. Earlier studies have confirmed that apoptosis occurs within the edge of the torn supraspinatus tendon, and in vitro studies on human tenocytes and chondrocytes show that TNF-α exerts an influence on this process. However, this is the first work to present the capacity of human supraspinatus tenocytes to prevent both the apoptotic and inflammatory processes by the increased expression of antiapoptotic Bcl-2 and anti-inflammatory IL-10 molecules. Research by Millar et al confirms that the tenocytes of the torn supraspinatus tendon have the capacity to produce TNF-α and also notes a positive correlation between TNF-α expression and caspase 8 concentration. The TNF-α cytokine is associated with the capacity to stimulate tenocyte apoptosis. The positive correlation between the increased expression of TNF-α and caspase 8 suggests that the former plays a role in activating the extracellular apoptosis pathway in the torn tendon.

Our study shows the similarity of dynamics of the homeostatic regulation of apoptotic and inflammatory
processes by human tenocytes at the torn supraspinatus tendon edge at the molecular level. However, a statistically significant ($P < 0.05$) trend could be seen regarding the balance of proinflammatory and anti-inflammatory agents across the 1-cm sample taken from the torn supraspinatus (Table III). These findings indicate that the homeostasis between the apoptotic and inflammatory processes associated with the human tenocytes in the torn supraspinatus tendon is dynamic and is highly dependent on their distance from the edge in a proximal direction.

Moving away from the tear margin, the TNF-$\alpha$ activity appears to decrease, as does the caspase 8 concentration, whereas the IL-10 concentration increases, which blocks the proapoptotic effect of TNF-$\alpha$ and modulates some of the catabolic features associated with it. TNF-$\alpha$ is known to stimulate the tenocytes to produce IL-1$\beta$, which activates metalloproteinases degrading the extracellular matrix. A higher percentage of apoptotic cells within the edge of the torn tendon signifies poorer healing potential of the tissue and correlates with a greater number of disorders of the extracellular matrix of the tendon in the affected rotator cuff. Increased apoptotic activity within a torn tendon edge results in a reduced number of tenocytes, leading to impaired collagen synthesis and degradation of the extracellular matrix. Experimental studies by Zwierzchowski et al confirm

| Table I | Expression of proapoptotic and antiapoptotic caspases and molecules, as well as proinflammatory and anti-inflammatory cytokines, within parts of tendon edge |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Proapoptotic and antiapoptotic molecules and cytokines | Distal part of tendon edge | Middle part of tendon edge | Proximal part of tendon edge |
| Caspase 9 | Mean $\pm$ SD | 0.122 $\pm$ 0.022 | 0.073 $\pm$ 0.019 | 0.044 $\pm$ 0.009 |
| | Range | 0.089-0.159 | 0.051-0.114 | 0.029-0.059 |
| Caspase 8 | Mean $\pm$ SD | 0.080 $\pm$ 0.014 | 0.065 $\pm$ 0.012 | 0.052 $\pm$ 0.007 |
| | Range | 0.057-0.102 | 0.051-0.099 | 0.041-0.064 |
| Caspase 3 | Mean $\pm$ SD | 0.160 $\pm$ 0.028 | 0.080 $\pm$ 0.024 | 0.049 $\pm$ 0.013 |
| | Range | 0.103-0.205 | 0.040-0.125 | 0.033-0.074 |
| Bax | Mean $\pm$ SD | 0.151 $\pm$ 0.016 | 0.103 $\pm$ 0.009 | 0.087 $\pm$ 0.004 |
| | Range | 0.128-0.179 | 0.087-0.117 | 0.081-0.096 |
| Bcl-2 | Mean $\pm$ SD | 0.080 $\pm$ 0.010 | 0.130 $\pm$ 0.011 | 0.150 $\pm$ 0.015 |
| | Range | 0.061-0.098 | 0.107-0.144 | 0.122-0.175 |
| TNF-$\alpha$ | Mean $\pm$ SD | 0.152 $\pm$ 0.019 | 0.074 $\pm$ 0.029 | 0.034 $\pm$ 0.024 |
| | Range | 0.118-0.170 | 0.031-0.124 | 0.0183-0.096 |
| IL-10 | Mean $\pm$ SD | 0.041 $\pm$ 0.021 | 0.076 $\pm$ 0.032 | 0.137 $\pm$ 0.026 |
| | Range | 0.0183-0.087 | 0.0413-0.141 | 0.097-0.182 |

| Table II | Differences in expression of proapoptotic caspase 9, caspase 8, caspase 3, and Bax molecule; antiapoptotic Bcl-2 molecule; proinflammatory cytokine TNF-$\alpha$; and anti-inflammatory IL-10 between parts of supraspinatus tendon edge |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Comparison between parts of tendon edge | Proapoptotic and antiapoptotic molecules and proinflammatory and anti-inflammatory cytokines | Caspase 9 decrease | Caspase 8 decrease | Caspase 3 decrease | Bax decrease | Bcl-2 increase | TNF-$\alpha$ decrease | IL-10 increase |
| D/M | .0015* | .08 | .0008* | .0004* | .0004* | .008* | .028* |
| D/P | .0004* | .0015* | .0004* | .0004* | .0004* | .008* | .008* |
| M/P | .012* | .010* | .005* | .003* | .013* | .008* | .008* |

D, Distal; M, middle; P, proximal.
Decreases and increases indicate changes in expression from the distal part to the proximal part of the tendon edge. The values are derived from the Wilcoxon nonparametric rank sum test (where $P < .05$ is considered significant).
* Significant $P$ value.
that apoptosis exerts a negative impact on the mechanical properties of the meniscus because of a decrease in total collagen dry mass. These changes were also connected with a significant increase in collagenase 1 and a decrease in the tissue inhibitor of metalloproteinase TIMP-2.43 Shirachi et al34 confirm a positive correlation between the expression of type I collagen mRNA at the edge of the ruptured rotator cuff tendon and postoperative cuff integrity. These clinical and experimental studies indicate that an increased level of apoptosis and metalloproteinase secretion due to increased levels of proinflammatory cytokines may influence the mechanical properties of the rotator cuff and its biological potential to heal after reconstruction.2,22,25,34,40,41,43

According to a histopathologic study by Longo et al,23 there is no need to excessively freshen the torn edge because the macroscopically intact supraspinatus tendon is also degenerated. A similar conclusion was reached by Lee et al,22 who found no differences in the apoptotic cell profile, identified by TUNEL assay, between the biopsy material from the edge and a 1-cm-deep region proximal to the margin of the torn supraspinatus tendon. It should be stressed, however, that the sample used by Lee et al was biopsy material and no information was present concerning the depth of the biopsy. The study also did not include the size of the rotator cuff rupture. In a surface biopsy, the degree of apoptosis 1 cm from the tear edge may result from a significantly increased concentration of TNF-α in the bursa subacromialis.3,36 Lundgreen et al25 observed a higher apoptotic index, and Shindle et al35 reported an increase in degenerative lesions in the macroscopically healthy tendon of the subscapularis muscle in patients with a torn supraspinatus tendon. It cannot be ruled out that the condition of both lesions resulted from the exposure of the subscapularis tendon to the very high concentration of IL-1β and TNF-α in the bursa subacromialis accompanying the supraspinatus tendon tear.3,38 The findings of our study confirm those of Lee et al, who noted the presence of a higher activity of proapoptotic caspases 9, 8, and 3/7 in the distal part than in the proximal part of a 1-cm margin from the torn supraspinatus tendon edge; however, they were not statistically significant. It should also be stressed that Lee et al22 noted that the TUNEL,25 luminescence spectrometry, and spectrophotometry methods used in their study have some limits: They are semiquantitative methods as opposed to the fully quantitative real-time PCR assay used in our study. A study by Goutallier et al15 indicates that resection should include the whole area of macroscopic tendon lesions. However, because this creates a problem with postsurgical tension, they propose a special technique of muscle mobilization to compensate. Chillemi et al8 confirm that considerable histopathologic changes occur within the 5-mm distal part of a torn supraspinatus tendon edge and conclude that histopathologic techniques should be used routinely to assess the ability of the rotator cuff to heal.

When one is deciding on the radicality of the resection, a number of aspects must be taken into account. A key consideration is the possibility of repairing the tear, which can be performed easily by inserting temporary stitches after prior extensive mobilization of the rotator cuff and evaluation of tension during movements of the shoulder. The additional question arises as to whether the degenerative changes of the tendon at the cellular, structural, and molecular levels will disappear after attachment of a biologically and mechanically imperfect tendon stump. Ianotti et al18 report that tears tend to recur between the sixth and 26th weeks after reconstruction. Therefore, even though the changes are partly reversible, the time needed for recovery is not cogent with the time needed for healing, and so, resection may be a logical and significant factor in promoting the integration of the reinserted tendon. Previous publications2,8,22,24,25,27,28,34,40,41 and our results suggest that during resection of the torn supraspinatus tendon edge, at least the first distal 3 mm should be removed to reach an area of relative homeostatic balance between both proapoptotic and antiapoptotic influences, as well as proinflammatory and anti-inflammatory influences (Table III). Moreover, they indicate that a resection performed 4 to 7 mm from the edge of the torn supraspinatus tendon may better enhance the quality and durability of the healing.
process and is a reasonable compromise between the risk of tear recurrence due to leaving the tendon stump as it is, with its low biological and mechanical value, and the risk due to high tension of the repair after its excessive resection. Within this range, the surgeon may select a degree of resection that would achieve the best compromise for each individual case and provide more precise data with regard to the rehabilitation process.

It should be noted that the number of patients included in the study was limited because of the high risk of tension-free closure after the resection of 1 cm from the supraspinatus tear. However, the resection of 1 cm of the upper part of the supraspinatus is relatively safe and does not limit the effectiveness of the margin-convergence technique for repair of a U-shaped tear. Although earlier studies used groups of patients that were smaller or larger than the group in our study, none of them incorporated both strict homogeneous tear morphology and a homogeneous reconstruction technique or used full-thickness sample material from the “oldest” part of the tear, which was wider. The process of maintaining the homeostasis of inflammation by human tenocytes derived from a torn supraspinatus tendon needs further investigation; however, the TNF-α and IL-10 values seem to be representative.

Our study is the first to present a correlation between apoptosis and the inflammation of the torn supraspinatus tendon at the molecular level, pointing to the possibly crucial role of endogenous TNF-α in initiating an extracellular apoptotic pathway. The obtained results seem to confirm earlier reports by Lundgreen et al concerning a more complex mechanism consisting of endogenous and exogenous factors that initiate the apoptosis of a torn rotator cuff.

This study highlights the potential of an endogenous mechanism blocking the apoptotic and inflammatory processes within human supraspinatus tenocytes, as well as the possibility for medication being used to block them during the preoperative and postoperative periods. TNF-α inhibitors are already successfully being used in rheumatoid arthritis, and a recent study confirms the possibility of using antioxidants, particularly anthocyanins, to exert an antiapoptotic effect on rotator cuff tenofibroblasts exposed to an oxidative stressor whereas another study used substance P to block the apoptosis of human tenocytes activated by TNF-α. These results encourage further research and suggest that the molecular enhancement of the rotator cuff healing process is a significant future prospect.

Conclusions

The human tenocytes within 1 cm of the edge of a torn supraspinatus tendon have an endogenous potential to reduce the expression of proapoptotic caspases 3, 8, and 9 and the Bax molecule, as well as the proinflammatory cytokine TNF-α, by increasing the expression of the antiapoptotic molecule Bcl-2 and the anti-inflammatory cytokine IL-10 in a distal to proximal direction. TNF-α may play a pivotal role in activating the extracellular pathway of tenocyte apoptosis at the torn supraspinatus tendon edge. The resection of the distal 3 mm of the torn supraspinatus tendon edge eliminates the part of highest proapoptotic and pro-inflammatory molecule expression. The resection of 4 to 7 mm of the torn supraspinatus tendon edge may enhance the healing process by achieving a reasonable compromise between the molecular potential of tenocytes to control the homeostasis of apoptotic and inflammatory processes and the mechanical aspect of the rotator cuff reconstruction.

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References


