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ORIGINAL ARTICLE

Association of synovial inflammation and inflammatory mediators with glenohumeral rotator cuff pathology

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Hypothesis: We hypothesized that patients with full-thickness rotator cuff tears would have greater synovial inflammation compared with those without rotator cuff tear pathology, with gene expression relating to histologic findings.

Methods: Synovial sampling was performed in 19 patients with full-thickness rotator cuff tears (RTC group) and in 11 patients without rotator cuff pathology (control group). Cryosections were stained and examined under light microscopy and confocal fluorescent microscopy for anti-cluster CD45 (common leukocyte antigen), anti-CD31 (endothelial), and anti-CD68 (macrophage) cell surface markers. A grading system was used to quantitate synovitis under light microscopy, and digital image analysis was used to quantify the immunofluorescence staining area. Quantitative polymerase chain reaction was performed for validated inflammatory markers. Data were analyzed with analysis of covariance, Mann-Whitney *U*, and Spearman rank order testing, with significance set at $\alpha = .05$.

Results: The synovitis score was significantly increased in the RTC group compared with controls. Immunofluorescence demonstrated significantly increased staining for CD31, CD45, and CD68 in the RTC vs control group. CD45+/68– cells were found perivascularly, with CD45+/68+ cells toward the joint lining edge of the synovium. Levels of matrix metalloproteinase-3 (MMP-3) and interleukin-6 were significantly increased in the RTC group, with a positive correlation between the synovitis score and MMP-3 expression.

Conclusions: Patients with full-thickness rotator cuff tears have greater levels of synovial inflammation, angiogenesis, and MMP-3 upregulation compared with controls. Gene expression of MMP-3 correlates with the degree of synovitis.

Level of evidence: Basic Science Study; Molecular Biology

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Keywords: Rotator cuff; synovium; synovitis; inflammation; cytokine; MMP

Rotator cuff tears are the most common cause of shoulder disability in the upper extremity[8] and account for most of the 4.5 million annual office visits for shoulder pain in the USA.[32] Extrinsic and intrinsic processes have both been proposed as the underlying cause of rotator cuff tears, but the

The Standard University Institutional Review Board approved this study (Stanford IRB Protocol 30479).

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exact etiology leading to the disease pathology remains unknown.[10,35] According to Neer,[30] impingement of the rotator cuff on the undersurface of the acromion can cause abrasion and tearing. Others have emphasized the role of intrinsic tendon degeneration caused by a variety of factors, including systemic patient factors and inflammatory mediators.[16,24,31]

Chronic inflammation has long been known to be a contributing factor in pathologies such as cardiovascular disease,[18,26] chronic gingivitis,[17,29] and rheumatoid arthritis.[4] Historically, chronic inflammation as a source of joint pain has been thought to encompass only the inflammatory arthropathies. Chronic inflammation has more recently been recognized as a source of joint pain and dysfunction in those with pathologies previously considered purely degenerative.[37,38]

Although many animal and basic science studies have investigated inflammatory mediators in rotator cuff pathology,[1,5,11,15,27] the study of this condition in an in vivo model has been limited because control samples have often been obtained from adjacent, nondiseased tendon within the same shoulder in those with known rotator cuff pathology[6,7,36,40] or from cadaveric specimens.[24] Other studies have examined only the synovial fluid[34,49] or the subacromial bursa,[3,45] often with conflicting results. Because the synovium is a major location for the production of inflammatory mediators, it is critical to understand the association between synovial inflammation and the generation of proinflammatory molecules to better understand the mechanisms behind the development rotator cuff pathology.

No prior studies have examined and correlated the amount of synovial inflammation with inflammatory mediator production in patients with full-thickness rotator cuff tears vs those with arthroscopically normal rotator cuff tissue. We hypothesized that patients with full-thickness rotator cuff tears would demonstrate increased synovial inflammation on microscopy and increased inflammatory mediators on gene expression analysis compared with those without rotator cuff pathology.

Methods

The study enrolled 30 patients. Inclusion criteria consisted of a history of nontraumatic onset of shoulder pain of >6 months and age ≥18 years. The study excluded patients with a history of inflammatory arthritis, prior surgery to the involved shoulder, previous trauma coincident with the onset of shoulder pain, those receiving glucocorticoid or other intravenous/intramuscular anti-inflammatory medication within 6 weeks of surgery, and those receiving oral anti-inflammatory medication within 2 weeks of surgery.

Patients were divided into 2 groups. The control group consisted of those with shoulder pain with no radiographic glenohumeral arthritis (Weinstein grade I) as well as an intact rotator cuff confirmed by magnetic resonance imaging (MRI) and arthroscopy. The rotator cuff tear (RTC) group consisted of patients without radiographic glenohumeral arthritis (Weinstein grade I) but with MRI and arthroscopically confirmed full-thickness rotator cuff tear.

All patients underwent surgical treatment by a single surgeon, with a synovial biopsy specimen obtained from a common site within the rotator interval after anterior portal establishment. A diagnostic arthroscopy was performed in all patients to ensure there were no nonbiopsy location sites that demonstrated nonrepresentative areas of abnormal synovitis.

The preoperative MRI was examined for tear size and the degree of fatty infiltration using the MRI modification of the Goutallier classification.[12] Tear size was determined in the anterior-posterior (AP) dimension and by the amount of retraction. AP tear size was determined on sagittal oblique T2 sequences, and the largest dimension noted in medial-to-lateral scrolling was recorded. The amount of tendon retraction was determined on coronal oblique T2 sequences and was recorded as the maximum distance of any rotator cuff tendon edge during anterior-to-posterior scrolling as measured from the medial aspect of the rotator cuff footprint immediately adjacent to the humeral head articular cartilage. All measurements were made using the IntelliSpace 4.4 digital picture archiving and communication system (Phillips, Amsterdam, The Netherlands).

Light microscopy analysis

Biopsy specimens were frozen at −80°C in optimum cutting temperature compound (Tissue-Tek, Torrance, CA, USA), and 10-μm cryosections were cut and affixed on glass slides. Sections from 3 different depths of the sample were used to obtain a representative sample of the entire specimen.

Tissue was stained with hematoxylin and counterstained with eosin, as previously detailed.[23] Light microscopy was used to calculate a synovitis score, which has been previously described and validated.[20,21] The synovitis score consists of 3 components—lining cell layer, synovial stroma, and inflammatory infiltrate—each graded on a scale of 0 to 3 points. Scores are added to achieve a final synovitis score. Samples were blinded and scored twice by 2 observers.

Immunofluorescence analysis

Cryosections were cut and placed on slides, as described above. Slides were fixed with 4% paraformaldehyde in 1× phosphate-buffered saline. Blocking was performed with 1% bovine serum albumin and 1% normal goat serum. Slides were incubated with monoclonal anti-human CD31 (Thermo Fisher Scientific, Waltham, MA, USA) and anti-human CD45 antibodies (BioLegend, San Diego, CA, USA) overnight, followed by secondary conjugated antibodies of anti-mouse immunoglobulin (Ig)G1 and IgG2a, respectively (Alexa Fluor 555 and 488; Life Technologies, Carlsbad, CA, USA). Another set of slides was incubated with monoclonal anti-human CD45 and anti-human CD68 antibodies (Abcam, Cambridge, UK), followed by conjugated secondary antibodies of anti-mouse

IgG2a and IgG2b, respectively (Alexa Fluor 555 and 488). A third set was incubated with monoclonal anti-human matrix metalloproteinase 3 (MMP-3) antibodies (BioLegend, San Diego, CA, USA). All slides were mounted with anti-fade gold containing 4′,6-diamidino-2-phenylindole (Promega, Madison, WI, USA) for visualization of nuclei. Slides were examined under a confocal laser microscope (Zeiss, Göttingen, Germany). ImageJ image analysis software (National Institutes of Health, Bethesda, MD, USA) was used to calculate immunostaining area for CD31, CD45, and CD68. Three separate areas of maximal staining were identified using the confocal microscope. The total area of pixel representation was recorded for each wavelength to assess the staining area for each image.

Quantitative polymerase chain reaction analysis

Human synovial tissue obtained at the time of surgery was immediately placed in RNAlater (Thermo Fisher, Carlsbad, CA, USA) and stored at −80°C until processing. Total RNA was extracted according to the manufacturer's instructions using the RNeasy Kit (Qiagen N.V., Venlo, Netherlands) and TRIzol reagent (Thermo Fisher). To generate complimentary DNA (cDNA), 1-μg total RNA was reverse transcribed using a high-capacity cDNA reverse-transcriptase kit (Applied Biosystems, Foster City, CA, USA). All cDNA samples were aliquoted and stored at −80°C.

The fold-change in messenger RNA (mRNA) expression was determined using TaqMan (Applied Biosystems) gene expression assays. Validated gene primers and TaqMan probes were obtained for MMP-3, MMP-13, interleukin (IL)-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, vascular endothelial growth factor (VEGF), and glyceraldehyde 3-phosphate dehydrogenase. These primers were chosen based on prior experience with inflammatory mediators in our laboratory as well as published results in other investigations.[10,36,40] Quantitative polymerase chain reaction experiments were run on the same day in triplicate and were normalized to glyceraldehyde 3-phosphate dehydrogenase using the delta (Δ) method.

Statistics

An a priori power analysis with preliminarily data was performed using our primary outcome measure, synovitis score, and our secondary outcome measure, MMP-3 expression. With standardized assumptions of $\alpha = .05$ and calculated effect sizes of 2.0 and 1.9 for the primary and secondary outcome measures, respectively, we required 6 patients in each group to achieve 80% power and 8 patients in each group for 95% power. Data were checked for normality using the Shapiro-Wilk test (SPSS 22; IBM Corp, Armonk, NY, USA). The synovitis score was calculated as the average of the 4 scores and compared using an analysis of covariance (ANCOVA). Intraobserver and interobserver reliability was assessed with the Cohen κ statistic. A pooled mean was calculated for immunofluorescence data and then analyzed with ANCOVA for between-group comparisons. Quantitative polymerase chain reaction was compared using the Mann-Whitney *U* test and an ANCOVA after log(10) transformation of data to control for covariates and non-normality of data, respectively. Correlation was examined with the Spearman rank correlation coefficient. Values are reported as mean ± standard deviation. An $\alpha = .05$ was set as significant.

Results

A total of 30 patients (19 RTC group, 11 control group) agreed to participate and were enrolled in the investigation. No patient who was offered enrollment declined to participate. Average age was 55 ± 11 years (range, 33-69 years; Table I). All patients in the RTC group had arthroscopically confirmed full-thickness rotator cuff tears. Sixteen patients in the RCT group underwent arthroscopic repair of their rotator cuff tear, 2 underwent débridement and biceps tenodesis for unrepairable massive rotator cuff tears, and 1 patient underwent a reverse total shoulder arthroplasty. MRI characteristics of the rotator cuff tears are detailed in Table I.

The synovitis score was significantly increased in the RTC group (3.6 ± 0.9) vs the control group (1.3 ± 1.0, $P < .001$;

Table I Demographic and surgical characteristics of those with rotator cuff tears vs patients without rotator cuff pathology

Group	Age	Procedure (number of patients)		AP tear size	Retraction	Goutallier classification
	(y)	Primary	Secondary	(mm)	(mm)	
Control	39.3 ± 11.7	Labral pathology (7)	–	–	–	–
		SAD/DCE (3)	–			
		AC reconstruction (1)	–			
RTC	60.4 ± 6.2*	RTC repair (16)	SAD (8)	18.6 ± 9.0	23.0 ± 10.2	2.4 ± 1.2
		BT (2)	BT (6)			
		Reverse TSA (1)	Tenotomy (3)			
			DCE (1)			

AC, acromioclavicular; *AP*, anterior-posterior; *BT*, biceps tenodesis (subpectoral); *DCE*, distal clavicle excision; *RTC*, rotator cuff tear; *SAD*, subacromial decompression; *TSA*, total shoulder arthroplasty.

* $P = .014$.

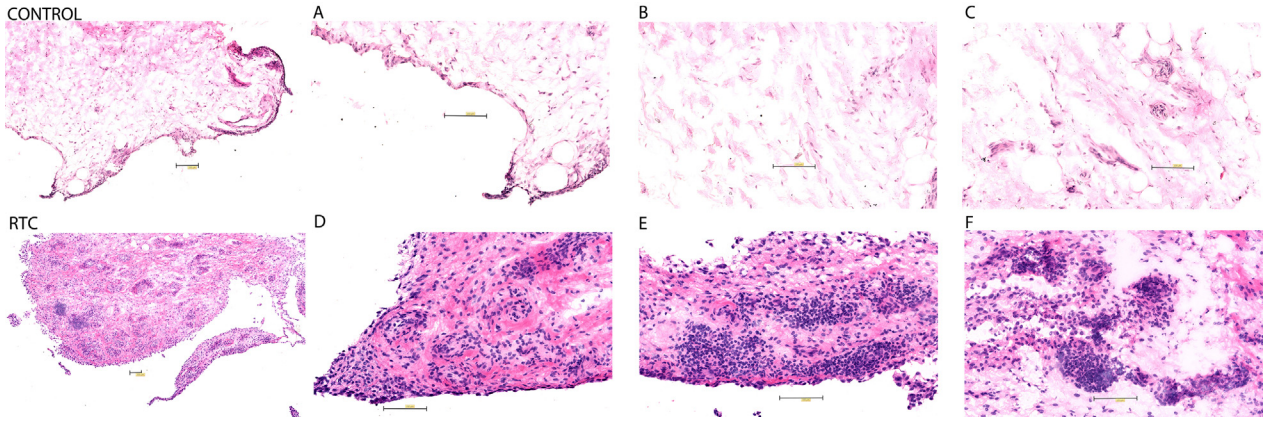

Figure 1 Light microscopy image of control and rotator cuff tear (*RTC*) group synovial biopsy specimens as well as higher magnification images demonstrating increased (**A and D**) synovial lining thickness, (**B and E**) stroma activation, (**C and F**) and cellular infiltrate in the RTC group. Scale bar = 100 μm; hematoxylin and eosin stain.

Fig. 1). Immunofluorescence demonstrated significantly increased staining for CD 31 ($P = .003$), CD 45 ($P < .001$), and CD68 ($P = .002$) in the RTC group (Figs. 2 and 3). CD45+/68– cells were noted perivascularly, with CD45+/68+ cells toward the joint lining edge of the synovium (Fig. 2, *B*, *box H*).

Univariate analysis of 8 predefined inflammatory mediators revealed a significantly increased level of MMP-3 ($P = .041$) and IL-6 ($P = .046$) gene expression in the RTC vs control groups, with a trend toward significance for IL-8 ($P = .087$; Figs. 4 and 5). The other inflammatory mediators (except VEGF) demonstrated nonsignificant trends for increased expression in the RTC group (Fig. 4). Immunofluorescent staining for MMP-3 revealed increased intensity in the RTC group samples, confirming results obtained from gene expression analysis (Fig. 6).

A positive correlation ($\rho = 0.356$) between the synovitis score and MMP-3 gene expression was observed; however, no correlation was found between the synovitis score and tear size or Goutallier classification nor between MMP-3 levels and AP tear size, amount of retraction, or Goutallier classification for those with rotator cuff tears.

Discussion

In this investigation, we found significantly increased synovial inflammation in those with full-thickness rotator cuff tears compared with those with intact rotator cuffs. Inflammatory cells demonstrated differentiation from a common white blood cell (CD45+/68–) toward a more specific macrophage lineage (CD45+/68+) as they moved from the perivascular area toward the joint lining surface. We also found that synovial inflammation correlated with synovial expression of MMP-3. These results regarding the association of synovial-based inflammation with rotator cuff disease (vs those without rotator cuff pathology) and the correlation between the histologic and gene expression analysis has not been previously described.

There is evolving evidence suggesting inflammation within the torn rotator cuff and glenohumeral space of those with rotator cuff tear pathology.[9,13,34,36,40] Lo et al[24] were among the first to investigate the association of a wide variety of inflammatory mediators with rotator cuff tears. Using cadaveric specimens as controls, they examined the expression of a number of MMPs and tissue inhibitors of metalloproteinase directly within tendon tissue. They found increased levels of MMP-13 in tissue obtained from patients with full-thickness rotator cuff tears. In contrast to our findings, they reported decreased levels of MMP-3 mRNA levels in the RTC group, but a major point of difference was that they used cadaveric specimens as controls. Given that inflammatory mediators and the overall inflammatory profile of a joint can change rapidly, as well as the high prevalence of rotator cuff pathology with advancing age,[47] the use of cadavers for control specimens would seem to be suboptimal and might confound results.

Similar to our study, recent investigations have suggested synovial inflammation in patients with rotator cuff tears. Gotoh et al[14] examined gene expression for IL-1β in synovial tissue taken from patients with full- and partial-thickness rotator cuff tears and found increased expression in those with full-thickness tears. Shindle et al[40] examined the inflammatory profile between patients with full-thickness rotator cuff tears as well as those with partial-thickness tears that were large enough to warrant take down and subsequent repair. They reported increased synovial inflammation and increased gene expression for IL-1β and IL-6 in the synovium of the full-thickness tear group. The authors also noted a more significant correlation between IL-1β and MMP levels in this group, suggesting that IL-1β may be a significant driver of MMP expression in rotator cuff pathology.

The Shindle et al[40] group and the current investigation both found increased levels of synovial inflammation in the full-thickness tear group; however, further comparisons are limited because our control groups differed. Rather than using patients with partial-thickness tears large enough to warrant take

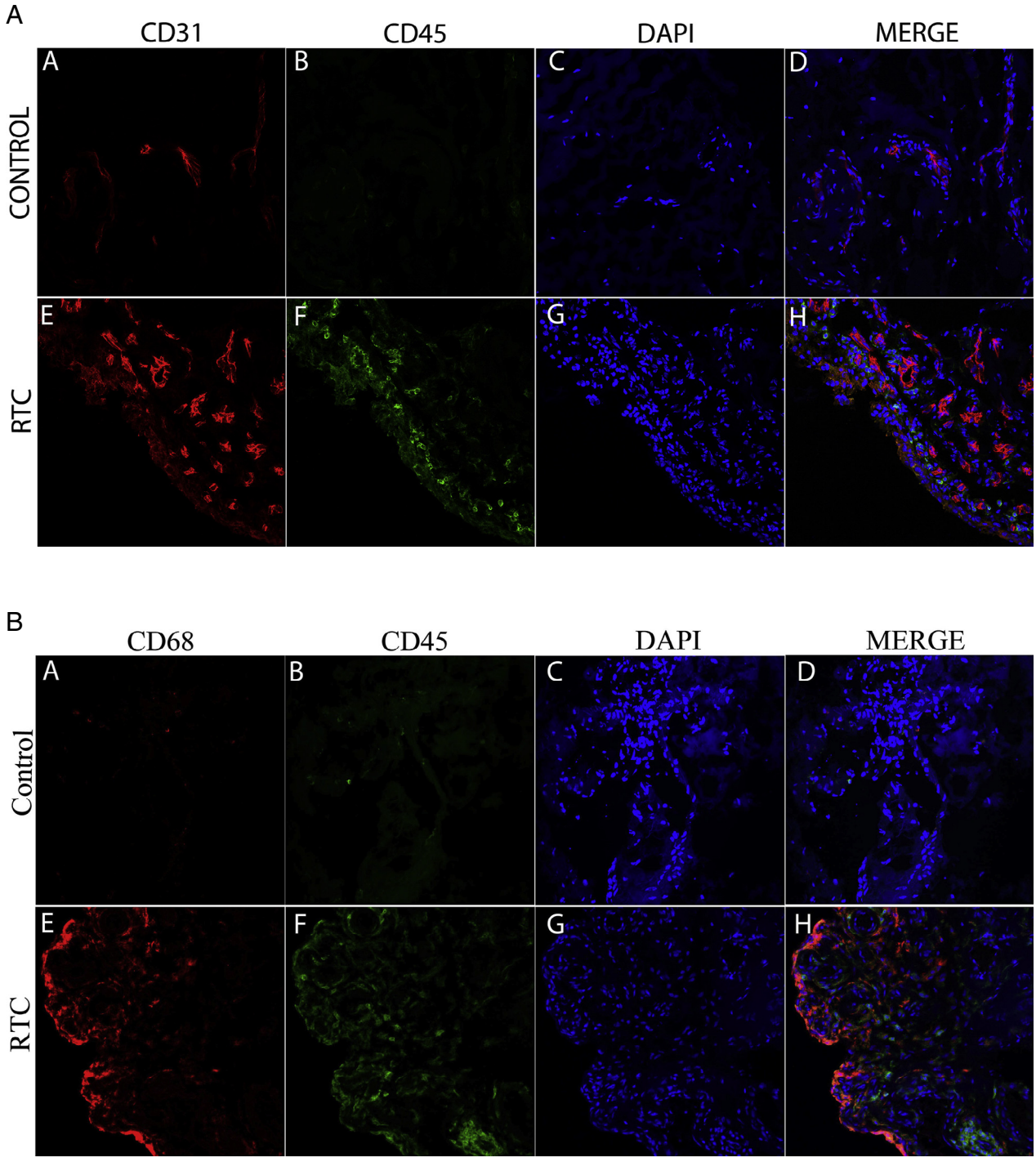

Figure 2 (**A**) Confocal laser microscope images of indirect immunofluorescence staining for CD31 (*A* and *E*), CD45 (*B* and *F*), 4′,6-diamidino-2-phenylindole (*DAPI*) (*C* and *G*), and merged image (*D* and *H*) for the control and rotator cuff tear (*RTC*) groups. Note the increased staining intensity in rotator cuff tear sections for CD31 (*E*) and CD45 (*F*). (**B**) Confocal laser microscope images of indirect immunofluorescence staining for CD68 (*A* and *E*), CD45 (*B* and *F*), DAPI (*C* and *G*), and merged image (*D* and *H*) for the control and the RTC group.

down and repair as a comparison group, we used surgical patients with macroscopically normal rotator cuff tissue.

Although the inciting events leading to the increased inflammation has not been definitively identified, evidence suggests that the initial insult may be a recognized or unrecognized minor trauma leading to a response of the innate immune system to damage associated molecular patterns,[42] which include products of tissue damage that are recognized by the host immune system and may include damaged intra-articular products such as collagen, fibronectin,

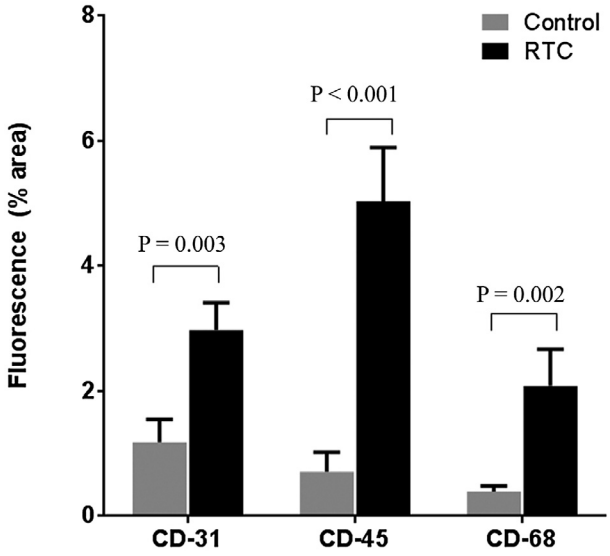

Figure 3 Bar chart depicts the average percentage fill for indirect immunofluorescence as seen under confocal laser microscopy for CD31, CD45, and CD68. Significant differences were noted in CD31, CD45, and CD68 staining. The *error bars* show the standard deviation. *RTC*, rotator cuff.

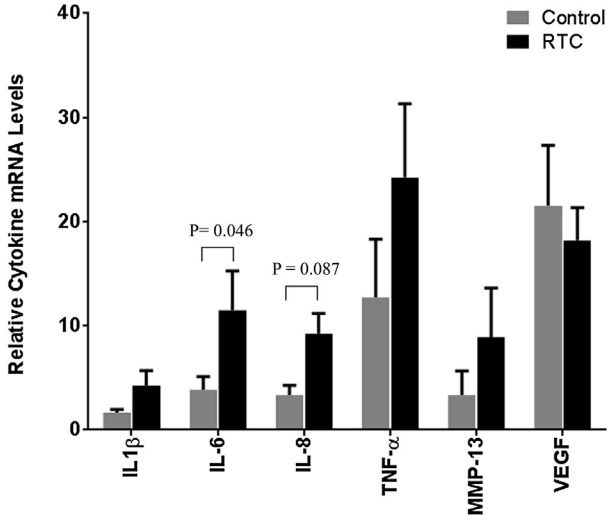

Figure 4 Bar chart represents the Δ value (with glyceraldehyde 3-phosphate dehydrogenase as the reference) for inflammatory mediator gene expression for interleukin (*IL*)-1β, IL-6, IL-8, tumor necrosis factor (*TNF*)-α, matrix metalloproteinase (*MMP*)-13, and vascular endothelial growth factor (*VEGF*). Significant difference in messenger RNA (*mRNA*) expression was noted in IL-6 with univariate analysis, with increased levels of other inflammatory mediators for the rotator cuff tear (*RTC*) group in all except VEGF. The *error bars* indicate the standard deviation.

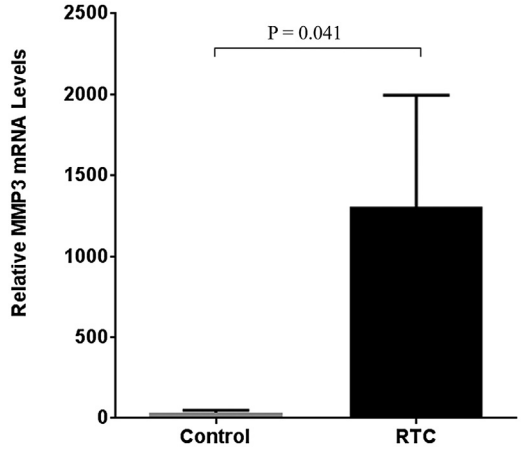

Figure 5 Bar chart of Δ values for messenger RNA (*mRNA*) expression of MMP-3 within control and rotator cuff tear (*RTC*) groups. There was significantly increased expression in the RTC group vs control group with nearly all Δ values for patients in the RTC group being higher than those without rotator cuff pathology.

hyaluronic acid, and biglycan.[42] These damaged products bind to one of the many pattern-recognition receptors of the innate immune system and set off a complex cascade of events that lead to activation of nuclear factor κ–light-chain-enhancer of activated B cells (NF-κB) and the production of inflammatory mediators such as IL-1, TNF-α, and IL-6, among others.[33,39,42] In immune cells, fibroblasts, and tenocyte cultures, these cytokines have been shown to induce a variety of MMP production, all of which accelerate the degradation of tendon extracellular matrix.[43,44,48]

In addition to prior studies suggesting a role for synovial inflammation in rotator cuff tear pathology, this study is the first study, to our knowledge, to compare the synovium and inflammatory profile of those with rotator cuff pathology vs a "control" group with arthroscopically normal rotator cuff tissue. Other investigators, mostly examining the inflammatory profile of synovial fluid (rather than synovium) in rotator cuff tears, have reported results similar to this investigation's findings.[34,49]

Yoshihara et al[49] found that elevated levels of MMP-3 in synovial fluid of patients with massive rotator cuff tears compared with those with partial or isolated supraspinatus pathology. In contrast to our findings, however, they were able to demonstrate a significant correlation between tear size and MMP-3 gene expression.

Osawa et al[34] also reported increased levels of MMP-3 in synovial fluid of patients with full-thickness rotator cuff tears compared with a small cohort of patients without significant rotator cuff pathology. However, the source of this increased MMP-3 has not been clarified, and our correlation of synovial inflammation with MMP-3 production suggests that synovial-based inflammation may contribute to the propagation of rotator cuff tear pathology.

Also unique to our investigation is the use of indirect immunofluorescence to quantify and localize inflammatory cells present within the synovial tissue of those with and without full-thickness rotator cuff pathology. We found significantly increased staining for myeloid (CD45/CD68) and endothelial cells (CD31) in the synovium of those with rotator cuff pathology vs controls. CD31, otherwise known as platelet

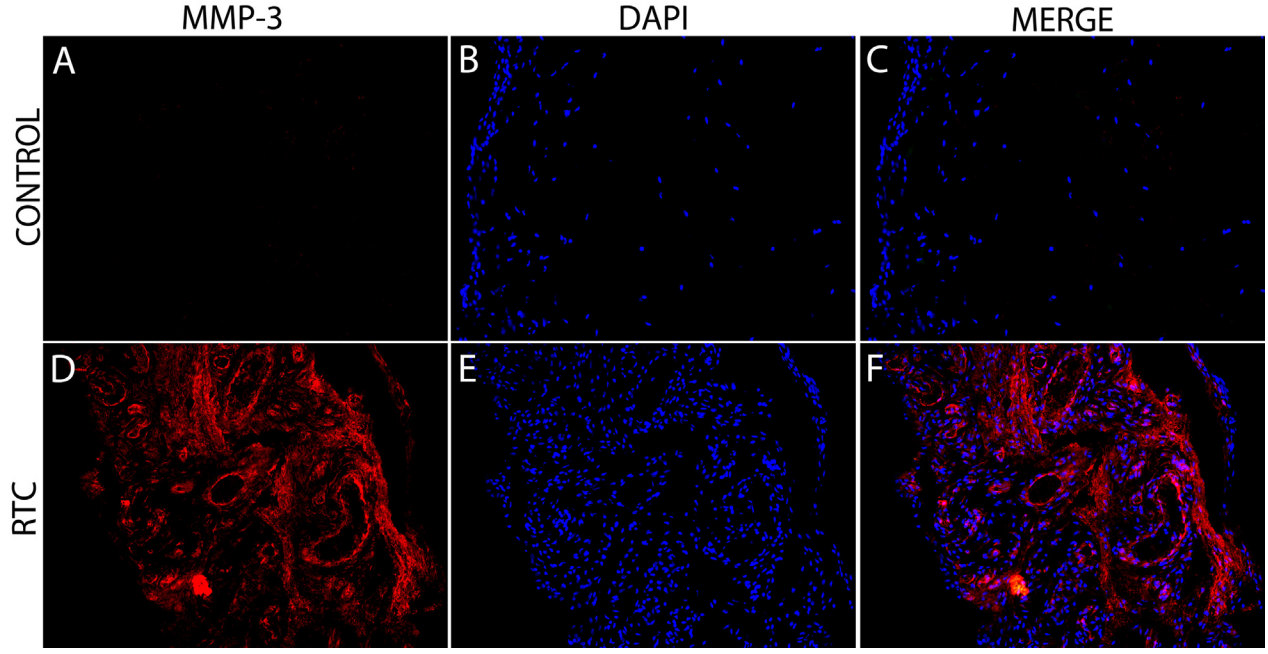

Figure 6 Confocal laser microscope images of indirect immunofluorescence staining for (**A and D**) matrix metalloproteinase 3 (*MMP-3*), (**B and E**) 4′,6-diamidino-2-phenylindole *DAPI*, and (**C and F**) merged image for the control and the rotator cuff tear (*RTC*) group.

endothelial cell adhesion molecule, makes up a large portion of endothelial cell intracellular junctions and thus represents the presence of vascularity.[46] Increased vascularity is a hallmark of the inflammatory process because it brings additional cells to the site of inflammation, and in the case of synovium, allows cells normally found exclusively in the plasma to enter the intra-articular space, further propagating the inflammatory process.[41,42]

The presence of increased CD45, also known as leukocyte common antigen, as well as CD68, a macrophage-specific marker, supports the premise of increased vascularity leading to the increased presence of inflammatory cells. We also observed an interesting pattern of CD45+/68– cells perivascularly with what appears to be macrophage differentiation (CD45+/68+) as cells migrate toward the joint lining edge of the synovium (Fig. 2, *B*, *box H*).

Our findings and investigations mentioned previously[14,24,40,49] show that MMPs may play a central role in rotator cuff disease pathology. The MMPs are a family of zinc-dependent enzymes that have the ability to degrade nearly all components of the extracellular matrix.[28] In general, MMPs can be divided into 4 main groups: stromelysins (MMP-3 and -10), collagenases (MMP-1, -8, and -13), gelatinases (MMP-2 and -9), and membrane-type MMPs (MMP-14). The stromelysins degrade proteoglycans, fibronectin, and collagen types III, IV, and V and also have an important role in the regulation of other MMPs, particularly MMP-1.[10] The collagenases are able to cleave nearly all types of collagen, including the triple-helix fibrillar collagens that provide structural integrity to tendons and other tissues.[2,25] Baseline production of MMPs is low but can be stimulated by other inflammatory mediators such as IL-1, IL-6, and TNF-α.[19,22,43]

Some limitations of the current investigation may have been related to sampling variability between patients. This is reduced, however, because a single surgeon obtained all biopsy samples. In addition, synovial inflammation may not be constant across time points and may also vary in response to external stimuli. All of our patients, however, underwent the same preoperative regimen on the day of surgery, and all biopsy specimens were taken at the same time during the procedure (after establishment of the anterior working portal), thus limiting variability.

Furthermore, our investigation may be underpowered to detect significant differences in our nonprimary outcomes, including measurement of inflammatory gene expression. Our a priori power analysis was aimed at the synovitis score, and we met our enrollment criteria for these outcomes measures.

We were not able to show significant differences between all inflammatory mediators, such as previously observed elevations in MMP-13 and IL-1β; however, difficulty estimating exact effect sizes may have resulted in a lack of power for some biomarkers.

Also, there was a significant difference in age between the 2 groups. Although this does introduce a possible confounder, age is not a known independent risk factor for synovial inflammation and reflects the typical timeline of increasing rotator cuff pathology as age increases.

Lastly, although we cannot definitively conclude a cause-and-effect relationship between rotator cuff tearing and increased synovial inflammation, it should be noted that those in the control group also had tearing or degeneration, or both, of other tissue (labrum and acromioclavicular joint) that did not lead to a robust inflammatory response.

The relatively small sample size required to observe significance for MMP-3 provides evidence for the potential role of MMP-3 in the development or, or both, propagation of rotator cuff pathology. Although prior studies have not reported increased MMP-3 levels in the setting of full-thickness rotator cuff tears, these studies often used tendon tissue, synovial fluid, or synovium from full thickness tears compared with those with partial-thickness tears.[36,40,49] Given the evidence for MMP-3 as not only a protease but also a regulator of other MMP activity,[10] this may represent an early stage within the inflammatory cascade that leads to activation of other MMPs and propagation of rotator cuff tendon destruction.

Conclusion

We observed robust synovial inflammation and increased inflammatory gene expression in those with full-thickness rotator cuff tears vs those without rotator cuff pathology. These data identify the synovium as a potential player in the pathogenesis of rotator cuff pathology and may guide the development of therapies to reduce pain and prevent progression of pathology associated with RCT disease.

Disclaimer

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