Histological and biochemical changes in a rat rotator cuff tear model with or without the subacromial bursa

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Research Article

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Abstract

Background

The subacromial bursa (SAB) plays an important role in the tendon healing process. Based on previous reports, co-culture of the rotator cuff (RC) and SAB have been shown to increase the expression of tendon-related genes and inflammatory cytokines, as well as to improve tensile strength in biomechanical evaluations. However, no studies have reported the nature of the biochemical changes occurring in vivo during tendon repair in the presence or absence of the SAB. In this study, we used a rat model of a full-thickness RC tear to determine how the presence or absence of the SAB alters the histological characteristics and gene expression in vivo in the injured RC.

Methods

The bilateral supraspinatus (SSP) tendons of rats were transected to create a full-thickness RC tear model. The right shoulder SAB was removed, and the left shoulder SAB was preserved. After 3 and 6 weeks, tissues were collected for histological (hematoxylin and eosin staining, Masson's trichrome staining, immunohistochemistry) and real-time quantitative polymerase chain reaction (RT-qPCR) evaluations.

Results

Histological results revealed greater cell density (3 weeks), neovascularization within the repaired tendon (6 weeks), and tendon thickening (6 weeks) with SAB preservation. Immunostaining results revealed significant increases in type 3 collagen (COL3) expression at 6 weeks with SAB preservation. The RT-qPCR results showed that SAB preservation induced statistically significant increases in the expression of scleraxis, matrix metalloproteinase-13 (MMP-13), interleukin-1β (IL-1β), and inducible nitric oxide synthase (iNOS) at 3 weeks and significant increases in COL3, IL-10, and arginase-1 (Arg-1) at 6 weeks.

Conclusion

An RC tear undergoes more appropriate inflammatory and repair phases during the tendon repair process when the SAB is retained.

Background

The rotator cuff (RC), which is composed of the supraspinatus (SSP), infraspinatus, teres minor, and subscapularis muscles, is an important joint structure [1]. The subacromial bursa (SAB), which lies between the RC and the acromion, serves to protect the RC as the RC moves. In the shoulder joint, the RC
presses the humerus against the glenoid fossa to stabilize the joint. An RC tear therefore compromises the stability of the shoulder, making raising the shoulder impossible and causing pain.

The most common causes of RC injuries are aging, repeated mechanical irritation, and trauma [2]. In general, tendon tissue lacks appreciable numbers of cells and blood vessels and has low metabolic activity; therefore, the proper healing of damage tendons tends to be difficult [3]. In humans, the SSP tendons are particularly susceptible to injury and are characterized by poor blood flow in what is known as a critical zone located 1.5 cm proximal to the greater tubercle of the humerus [4]. For these reasons, once it is damaged, the SSP tendon does not readily undergo natural repair. Advances in implants and techniques have improved the outcomes of arthroscopic RC repair; however, postoperative retears remain a serious complication [5].

The SAB is considered a source of inflammatory cytokines and matrix metalloproteinase (MMP), which cause pain [6]. Therefore, the SAB is often resected or cauterized during RC repair [7]. However, the SAB is also a source of mesenchymal stem cells, suggesting that it also contributes to the repair of a torn RC [8]. An in vitro evaluation by Tamburini et al. showed greater expression of tendon-related genes (type 3 collagen [COL3] and decorin) and inflammatory cytokines after a 3-week co-culture of tendon and SAB tissue than after monoculture of tendon or SAB alone [9]. Sun et al., who conducted a biomechanical assessment to compare rat full-thickness RC tear models between SAB-preserved and SAB-removed groups, found that the repaired RC in the SAB-preserved rat model had statistically significant strength gains at both 3 and 9 weeks postoperatively [10]. At present, the benefits of preserving versus removing the SAB in the repair of RC tears remain unclear, but available evidence suggests that differences in the healing process may arise depending on the presence or absence of the SAB.

Tendon healing is characterized by overlapping phases of inflammation, repair, and remodeling [11]. In the inflammatory phase, a blood clot forms and pro-inflammatory cytokines attract inflammatory cells, such as neutrophils and macrophages, to the site of injury [12]. The macrophages are involved in the recruitment of fibroblasts and secretion of pro-angiogenic factors that stimulate the formation of new capillary networks, thereby promoting neovascularization and tissue repair within the wound [13]. During the repair phase, fibroblasts are the most common cell type and actively create a disorganized tissue at the injury site. The levels of COL3 and DNA are at their highest during this phase and aid in collagen synthesis, with COL3 gradually converted to type 1 collagen (COL1) [12, 14]. The remodeling phase, which begins 1–2 months after the injury and lasts for over a year, is primarily characterized by the involvement of COL1 [15]. However, the repaired tissue is unable to fully recover due to elevated water content and reduced levels of collagen quantity and quality [16].

Recent evidence now supports the involvement of two types of macrophages related to the repair phases: M1 macrophages and M2 macrophages [17]. The M1 macrophages release inflammatory cytokines, such as interleukin-1β (IL-1β) and IL-6, and inflammatory mediators, including inducible nitric oxide synthase (iNOS). By contrast, M2 macrophages release anti-inflammatory cytokines, such as IL-4, IL-10,
transforming growth factor-β (TGF-β), and arginase-1 (Arg-1). IL-13 induces the differentiation of macrophages into M2 macrophages, which then produce IL-10 and TGF-β to promote tendon healing [18].

The purpose of the present study was to investigate the role of the SAB in the tendon healing process using a rat full-thickness rotator cuff tear model at two time points (3 and 6 weeks) and examining the repair process both histologically and biochemically. The inflammatory phase was anticipated to predominate at 3 weeks and the repair phase at 6 weeks. Furthermore, preservation of the SAB was expected to increase the expression of inflammatory cytokines (MMP-13, IL-1β, IL-6, and iNOS) at 3 weeks, anti-inflammatory cytokines (TGF-β, IL-4, IL-10, and Arg-1) at 6 weeks, and tendon-related markers (COL1, COL3, decorin, tenomodulin, and scleraxis) throughout the 3–6 week investigation period.

Methods

Surgical methods for creating the rat full-thickness RC tear model

Approval for the study was obtained from the Animal Committee of Tokyo Medical and Dental University, and all animal care procedures were performed in accordance with the ARRIVE guidelines. Wild-type male Lewis rats (11 weeks old, n = 21) were used. Under isoflurane anesthesia, a skin incision was made with a scalpel. The deltoid muscle was split longitudinally to reach the SSP and SAB. The SAB was removed on the right side but preserved on the left side. A scalpel was used to transect 4 mm proximal to the humeral attachment of the SSP tendon [10]. The wound was closed by suturing the subcutaneous tissue and skin using 5 − 0 nylon thread (Fig. 1).

The rats were euthanized at postoperative weeks 3 and 6. The RC, SAB, humerus, scapula, and the other surrounding connective tissues were harvested and subjected to histological assessments, including hematoxylin & eosin (H&E) staining, Masson’s trichrome staining, and immunohistochemistry (COL3, decorin, and scleraxis; n = 6 / group). RNAs were also extracted from the SSP tendons and compared by real-time qPCR for the expression of COL1, COL3, tenomodulin, decorin, scleraxis, MMP-13, IL1-β, IL-6, iNOS, TGF-β, IL-4, IL-10, and Arg-1 (n = 3 per group per time point). An overview of the study is provided in Fig. 2.

H&E Staining

Deparaffinized tissue sections on slides were immersed in hematoxylin solution for 5 min, washed in running water, and then immersed in eosin solution for 3 min. The slides were washed with alcohol and immersed in xylene solution three times for 5 min each. The excess xylene was drained off, the tissue sections were mounted in Softmount (Wako, Tokyo, Japan), and coverslips were applied.

Masson’s Trichrome Staining
Deparaffinized tissue sections on slides were immersed in a mixture of 10% potassium dichromate and 10% trichloroacetic acid solution for 20 min. The sections were then rinsed with tap water and stained with Weigert's iron hematoxylin working solution for 10 min. After rinsing with tap water for 5 min, the sections were stained with a mixture of tungstophosphoric and molybdophosphoric acid for 1 min, followed by staining with orange G for 1 min. The slides were briefly placed in 1% acetic acid solution, followed by staining with Ponceau acid and fuchsin solution with azofloxin for 20 min, 2.5% tungstophosphoric acid for 10 min, and aniline blue for 5 min. The tissues were differentiated with 1% acetic acid after each staining. The slides were washed with alcohol and immersed in xylene solution three times for 5 min each. The excess xylene was drained off, the tissues were mounted in Softmount (Wako), and coverslips were applied.

**Immunohistochemistry**

For immunostaining of COL3, paraffin-embedded sections were treated with 200 µg/mL proteinase K (Dako, Glostrup, Denmark) for 15 min at room temperature. Immunostaining of decorin and scleraxis was conducted by immersing the slides in 10 mM Tris containing 1 mM EDTA (pH 9.0) and heating at 95°C for 1 h to retrieve antigens. The slides were immersed in methanol containing 0.3% H₂O₂ for 30 min and then washed with Tris-buffered saline containing 0.1% Tween-20 (TBS-T buffer). After blocking with 5% normal goat serum, the sections were incubated overnight at 4°C with antibody against COL3 (1:200; Bioss Inc., Woburn, USA), decorin (1:200; Bioss Inc.), or scleraxis (1:150; Abcam, Cambridge, United Kingdom). After washing three times with TBS-T, the sections were incubated with secondary antibodies conjugated with horseradish peroxidase (1:200; Abcam) for 1 h at room temperature. Diaminobenzidine (DAB) solution (Dako) was then applied for 5 min, and the cells were counterstained with hematoxylin. The DAB-positive and hematoxylin-positive areas were quantified using the Colour Deconvolution plugin for Fiji/Image J. The DAB-positive %area was determined for COL3 and decorin. For scleraxis, since it is localized in the nucleus, the DAB-positive area per hematoxylin-positive area was evaluated.

**Rna Extraction And Complementary Dna Synthesis**

The acquired SSP samples were preserved in RNAlater (Ambion, Foster City, USA), and then total RNA was extracted using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Complementary DNAs (cDNAs) were produced with the Transcriptor High Fidelity cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland).

**Real-time Quantitative Pcr (Rt-qpcr)**

The primers used are shown in Table 1, and each experiment was conducted in duplicate. Using SYBR Green dye (THUNDERBIRD Next SYBR qPCR Mix; Toyobo, Osaka, Japan), the fluorescence intensity was monitored using the LightCycler 480 instrument (Roche). The cycling conditions for PCR were as follows:
95°C for 1 min, 45 cycles at 95°C for 15 s, and 55–60°C for 30 s. GAPDH was used as a stable reference gene to normalize the expression.

Table 1. List of rat specific primers used for RT-qPCR

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer sequences (5'-3')</th>
<th>Product length</th>
</tr>
</thead>
<tbody>
<tr>
<td>COL1</td>
<td>Fwd: TCCTGCGGATGTCGCTATC Rev: CCAATGAGGCCTACGTGCTTGG</td>
<td>101</td>
</tr>
<tr>
<td>COL3</td>
<td>Fwd: AGTACA0CTGCGCCTCTCTCA Rev: TGTTTTTCGAGTGTGATGTAATGTTC</td>
<td>72</td>
</tr>
<tr>
<td>Tmmd</td>
<td>Fwd: CTACAGCAATGCGCAGAAGCAGAAAG Rev: GACCTACAAAGTAGATGCACAGTATAC</td>
<td>146</td>
</tr>
<tr>
<td>Dcn</td>
<td>Fwd: CACTCCAGAGCTCTGAC Rev: AGTGGGTTCTGCGCCAGTTC</td>
<td>106</td>
</tr>
<tr>
<td>Sce</td>
<td>Fwd: GAGACGGCGCGAGAAG Rev: TGGTCTCAA6TTCTCTGCTGGCT</td>
<td>73</td>
</tr>
<tr>
<td>MMP-13</td>
<td>Fwd: CTATGTCTGCCTAGCTCCTGTC Rev: CAACCCCTGTTACCTACCACCTTAT</td>
<td>85</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Fwd: AAAAGAAGAGATGGAACAGGCTGTT Rev: GAACTGTGCAGACTGAAACTC</td>
<td>80</td>
</tr>
<tr>
<td>IL-6</td>
<td>Fwd: TCCTACCCCAAACCTGATGGCTC Rev: TTGATTCTTTGCTTCCTTTGCC</td>
<td>79</td>
</tr>
<tr>
<td>INOS</td>
<td>Fwd: CACCAACCTCCTGTTCAAC Rev: CAAATCCAAACTGCTCCCAA</td>
<td>132</td>
</tr>
<tr>
<td>TGF-β</td>
<td>Fwd: CTGGCTGACGCCACTGATAC Rev: AGCCTGTATGCTGCTCT</td>
<td>94</td>
</tr>
<tr>
<td>IL-10</td>
<td>Fwd: GTTGCCAGCGTGTCAAGAAA Rev: TTCTGGGTATGTTGCTCT</td>
<td>78</td>
</tr>
<tr>
<td>Arg-1</td>
<td>Fwd: CACCTGAGTTTTGTATGGTATG Rev: TCCTGAAAGTTGGCTCTTCTTGT</td>
<td>135</td>
</tr>
</tbody>
</table>

Statistical analysis

Statistical analysis was performed using SPSS (IBM Corp., Chicago, IL, USA). The paired t-test was used for comparison between the two groups. Data were expressed as the average ± standard deviation (SD). A p-value < 0.05 was considered statistically significant.

Results

Histological observations

At 3 weeks, the gap between the SSP tendon and the humerus was connected to the humerus through a highly cellular interstitium in the SAB-preserved group (Fig. 3). By contrast, in the SAB-removed group, the gap was partially connected to the humerus through tendon-like tissue, but prominent cracks were evident between the fiber bundles, and the cellularity was less than in the SAB preserved group. By 6 weeks, the
gap between the SSP tendon and humerus contained thickened interstitium, as well as many blood vessels, in the SAB-preserved group. By contrast, in the SAB-removed group, the gap contained tendon-like tissue with oriented fiber bundles, and the tissue was thinner and less vascular than in the SAB-preserved group.

**Immunohistochemical Analysis**

At 6 weeks, COL3 was expressed throughout the repaired tendon in the SAB-preserved group, and the percentage of positive area was significantly higher in the SAB-preserved group than in the SAB-removed group (Fig. 4). Decorin and scleraxis expression were observed in the repaired tendons in both groups, and the percentage of positive areas was not significantly different between the two groups.

**Rt-qpcr Analysis**

The expression of 12 different genes in repaired tendons was compared between the two groups at 3 and 6 weeks. The expression of the 12 genes was also analyzed in normal tendons for reference, and was weaker than in the two groups with tendon transections in most cases at both 3 and 6 weeks (Fig. 5). At 3 weeks, scleraxis, MMP-13, IL-1β, and iNOS expression was significantly higher in the SAB-preserved group than in the SAB-removed group. At 6 weeks, COL3, IL-10, and Arg-1 expression was significantly higher in the SAB-preserved group than in the SAB-removed group.

**Discussion**

Histological evaluation revealed a higher cell density at 3 weeks and a greater formation of blood vessels within the torn SSP at 6 weeks in the SAB-preserved group than in the SAB-removed group. The SAB-preserved group also showed tendon thickening at 6 weeks. Inflammatory cytokines are expressed a few days after tendon rupture, resulting in cell proliferation [19], and some inflammatory cytokines, such as IL-1β and IL-6, are known to upregulate the expression of vascular endothelial growth factor, which is necessary for angiogenesis [20]. Rotator cuff tendinopathy increases angiogenesis within the SSP tendon, and may cause pain, but angiogenesis is also necessary for tissue repair [21].

Previous biomechanical evaluations of a full-thickness RC tear rat model at 3 and 9 weeks have revealed noteworthy thickening of the tendon, especially on the SAB side, following SAB-sparing operations, as well as a boost in strength when the SAB was preserved [10]. The histological results of the present study are consistent with these previous observations. Our findings suggest that SAB preservation following tendon injury induces histological changes that can promote the process of tendon repair by stimulating cellular proliferation around the site of the injury and promoting neovascularization, thereby resulting in greater tendon thickening.

Our results also indicated that SAB preservation promoted a significant increase in the expression of scleraxis, a tendon-related marker, at 3 weeks and in COL3, another tendon-related marker, at 6 weeks.
Scleraxis is a transcription factor that plays a crucial role in tendon development and repair processes while also regulating the expression of collagen and other extracellular matrix proteins in tendon cells [22]. The formation of an organized cellular bridge between the injured tendon stubs is a crucial step in the process of tendon healing [23], and scleraxis is involved in the immediate induction of tendon generation [24]. The extracellular matrix of normal tendons is composed primarily of COL1 (65–80%), while COL3 contributes to the structure of newly formed tissue during the early stages of tendon healing [25]. The transition between these two collagen types during the healing process of tendons has been shown by Amiel et al., who demonstrated a biochemical change from COL3 to COL1 in a rabbit model of knee medial collateral ligament injury [26]. During the remodeling phase, mechanical loading is required for the tendon to change the collagen fibers to COL1 [27]. An increased proportion of COL3 can significantly impact the structural integrity of the tendon, resulting in a decreased capacity to resist tensile forces and an increased risk of rupture under normal physiological loads [9, 28]. Our results did not indicate any difference in COL1 expression between groups, possibly because an evaluation period of 6 weeks may have been insufficient to observe the transition from COL3 to COL1. Our findings indicate that preservation of the SAB during the early stages of tendon healing is associated with an upregulated expression of several essential tendon-related markers implicated in the repair process. However, given the complex and dynamic nature of tendon remodeling, further investigation is necessary to establish the long-term effects of SAB preservation on overall tendon function and recovery.

In the present study, the SAB-preserved group exhibited a significant increase in the expression of inflammatory mediators (IL-1β, iNOS) at 3 weeks and anti-inflammatory mediators (IL-10, Arg-1) at 6 weeks. The expression of iNOS also tended to decrease from 3 to 6 weeks in the SAB-preserved group. The initiation of an appropriate healing process requires the induction of an adequate inflammatory response. During the inflammatory phase, M1 macrophages contribute to the production of IL-1β, IL-6, and iNOS, and this response has been suggested to inhibit tendon healing and further promote MMP production [29].

The repair phase is promoted by the induction of anti-inflammatory M2 macrophages, which secrete IL-10, a cytokine that suppresses the production of inflammatory mediators but is reportedly not expressed for 28 days after injury [30]. The M2 macrophages also express Arg-1, which exerts anti-inflammatory effects by suppressing iNOS production [31]. Therefore, the balance between iNOS and Arg-1 plays an important role in the activation of inflammatory processes and subsequent repair processes [32]. Our results indicate that the transition from the inflammatory to the repair phase in our rat model occurs around 3 to 6 weeks.

Our study has several limitations that must be addressed. First, the rats used in our study were not maintained at a constant resting level, which could have resulted in variations in gene expression due to physiological loading [33]. Moreover, our evaluation was restricted to a 6 week period; therefore, conducting further investigations extending beyond this timeframe would be beneficial, particularly if the timing extended to the remodeling phase. Finally, the study was conducted in rats; therefore, generalizability to humans is uncertain. Nevertheless, rats are known to share a high degree of genetic
homology with humans (80–90%) and exhibit similar bone morphology [34, 35]. Despite these limitations, our findings provide important insights into the potential role of SAB preservation in tendon healing and may have clinical significance. Further research using larger animal models and human subjects is necessary to obtain a better comprehension of the mechanisms underlying tendon healing and the impact of SAB sparing on long-term outcomes.

Conclusions

Preservation of the SAB resulted in higher cellularity in the torn rotator cuff in a rat model at 3 weeks and promoted angiogenesis at 6 weeks in the injured area. Immunostaining of rotator cuff regions revealed increased COL3 expression at 6 weeks. RT-PCR of rotator cuff regions showed increased scleraxis expression at 3 weeks and COL3 expression at 6 weeks. The levels of inflammatory cytokines increased at 3 weeks, and the levels of anti-inflammatory cytokines increased at 6 weeks.

Abbreviations

Arg-1: Arginase-1
COL: Collagen
DAB: Diaminobenzidine
GAPDH: Glyceraldehyde-3-phosphate dehydrogenase
H&E: Hematoxylin and eosin
IL: Interleukin
iNOS: Inducible nitric oxide synthase
MMP: Matrix metalloproteinase
RC: Rotator cuff
RT-qPCR: Real-time quantitative polymerase chain reaction
SAB: Subacromial bursa
SD: Standard deviation
SSP: Supraspinatus tendon
TGF: Transforming growth factor
Declarations

Ethics approval and consent to participate

All experiments and methods were conducted in accordance with relevant guidelines and regulations. All animal care and experimental protocols were performed in accordance with the ARRIVE guidelines and approved by the Animal Committee of Tokyo Medical and Dental University (approval number: A2022-150A).

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors’ contributions

YM designed the study, provided ideas, performed all experiments, organized the data, and wrote the manuscript. KE provided ideas and revised the manuscript. IS provided ideas and completed the manuscript. All authors have read and approved the submitted draft of the paper.

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References


Figures

Fig. 1
Figure 1

Surgical procedure used in the right shoulder in the rotator cuff full-thickness tear rat model. (a) A skin incision was made and extended through subcutaneous fat until the acromion and deltoid muscle were observed. (b) The deltoid muscle proximal to the acromion was longitudinally split, and the subacromial bursa (SAB) overlying the supraspinatus (SSP) tendon was identified. (c) The SAB on the right shoulder was gently removed, and the SAB on the left side was preserved. (d) The deltoid muscle distal to acromion was longitudinally split to identify the greater tubercle of the humerus. (e) The SSP was transected 4 mm proximal to the greater tubercle of the humerus.
Figure 2

Outline of the experiment. The bilateral supraspinatus (SSP) tendons of Lewis rats were surgically resected. The subacromial bursa (SAB) on the right shoulders was removed, and the SAB on the left was preserved. The SSP tendons were collected after 3 or 6 weeks and analyzed histologically and by the real-time quantitative polymerase chain reaction (RT-qPCR).
Figure 3

Histological analysis of supraspinatus (SSP) tendons. Representative images of the SSP tendon stained with hematoxylin and eosin (H&E) and Masson's trichrome are shown. Black arrows indicate the location of neovascularization. Black dotted lines indicate the margin of the SSP tendon.
Figure 4

Immunohistochemical analysis of supraspinatus (SSP) tendons. Representative images of immunostaining and quantitative analyses are shown. For COL3 and decorin, the diaminobenzidine (DAB)-positive area (% area) in SSP tendon was evaluated with Image J. For scleraxis, the DAB-positive area per hematoxylin-positive area in the SSP tendon was evaluated with Image J. Black dotted lines indicate the margin of the SSP tendon. Values are shown as mean ± standard deviation (n=6); p < 0.05.
Figure 5

RT-qPCR analysis of SSP tendons. Each graph represents the expression of a gene relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are expressed as mean ± SD (n=3); p < 0.05.

Supplementary Files
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- RCSupplementaryFig.1miura230317.pdf