Are the Symptoms of Calcific Tendinitis Due to Neoinnervation and/or Neovascularization?

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Investigation performed at the Orthopaedic Research Institute, St. George Hospital, University of New South Wales, Sydney, New South Wales, Australia, and the Institute of Infection, Immunity and Inflammation, College of Medicine, Veterinary and Life Sciences, University of Glasgow, Glasgow, United Kingdom

Background: Calcific tendinitis can be a substantial cause of pain and dysfunction in the shoulder, and the pathophysiology is unclear. Recent studies have shown a link among nerve ingrowth, neovascularization, and pain in tendinopathy. The aim of this study was to determine whether there is evidence of neoinnervation and/or neovascularization in calcific tendinitis lesions of the shoulder.

Methods: At arthroscopy, ultrasound was used to identify calcium within the tendon. Samples were taken from the supraspinatus tendon adjacent to the calcific lesion (in the calcific tendinitis group, with ten patients), the torn supraspinatus tendon of patients undergoing rotator cuff repair (the rotator cuff tear group, with ten patients), and the subscapularis tendon of patients undergoing a stabilization surgical procedure (the control group, with ten patients). Biopsied tendon samples were evaluated immunohistochemically by quantifying the presence of macrophages (using CD68 and CD206), T cells (CD3), mast cells (mast cell tryptase), vascular endothelium (CD34), and peripheral nerve markers (PGP 9.5).

Results: There was a twofold to eightfold increase of nerve markers, neovascularization, macrophages, M2 macrophages, and mast cells in the calcific tendinitis group compared with the rotator cuff tear group (p < 0.001) and the control group (p < 0.001). Increased nerve counts positively correlated with more frequent extreme pain (r = 0.5, p < 0.01) and with increased neovascularization (r = 0.7, p < 0.01) and counts of CD68 macrophages (r = 0.8, p < 0.01), M2 macrophages (r = 0.6, p < 0.01), and mast cells (r = 0.7, p < 0.01).

Conclusions: This is the first study to show a significant increase in neovascularization and neoinnervation in calcific tendinitis lesions of the shoulder along with an eightfold increase in mast cells and macrophages. The findings are consistent with the hypothesis that, in calcific tendinitis, the calcific material is inducing a vigorous inflammatory response within the tendon with formation of new blood vessels and nerves.

Clinical Relevance: This study helps to explain why calcific tendinitis is related to substantial pain in the clinical setting.

Calcific tendinitis was first described by Codman in 1907 and consists of the deposition of basic calcium phosphate crystals within the tendons of the rotator cuff, most commonly the supraspinatus tendon. The prevalence of calcification in the shoulder tendon was 2.7% in Bosworth’s series of 6061 office workers and 20% in Ruttimann’s radiographic series of 100 asymptomatic shoulders.

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Calcific tendinitis can present in three different clinical scenarios. It can be a painless asymptomatic incidental finding, a condition involving chronic low-grade pain, or a very painful condition that affects the range of movement and function of the shoulder. Pain is often aggravated by abduction of the arm above shoulder height or by lying on the affected shoulder. Calcium deposits within the rotator cuff are visible on radiographic and ultrasound imaging. The calcium appears as a radiopaque deposit within the rotator cuff, normally adjacent to the greater tuberosity of the humerus. With ultrasound imaging, the calcium within the rotator cuff is identified as hyperechoic material within the supraspinatus tendon.

Treatment for calcific tendinitis has included oral analgesics and nonsteroidal anti-inflammatory drugs, injections, barbotage, and lavage. Cortisone injections may be helpful when the shoulder is acutely inflamed. If conservative treatment does not alleviate the problem, a surgical procedure is another option.

The cause of calcific tendinitis is not yet understood. Other authors have suggested that calcific tendinitis may be attributed to ischemia, metabolic disturbances, and fibrocartilaginous transformations of the tendon tissue. Recent studies have highlighted neovascularization, nerve ingrowth, and chronic pain in patients presenting with tendinopathy, and other studies have found an increase in inflammatory markers (mast cells and macrophages) and neovascularization in patients with a tendinopathic supraspinatus compared with patients with an intact subscapularis.

The aims of this study were to evaluate neovascularization, neoinnervation, and inflammatory infiltrate in patients presenting with calcific tendinitis and to assess any correlation of these processes with patient symptoms.

**Materials and Methods**

**Clinical Evaluation**

This was a prospective cohort observational study of patients who underwent arthroscopic debridement of calcific tendinitis. The collection of human tissue was approved by the Human Research Ethics Committee of the South Eastern Sydney Local Health District (HREC 11/26 and HREC 96/55). Detailed clinical information, including patient age, duration of the disease, and symptoms of the disease, was recorded during the clinical consultation. Rotator cuff tendon samples were collected during arthroscopic rotator cuff repair or shoulder stabilization of patients without calcific tendinitis to serve as controls.

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<th>TABLE I Patient Demographic Characteristics and Clinical Details</th>
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*The values are given as the mean and the standard error.

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<th>TABLE II Histological Features in the Three Study Groups*</th>
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<td><strong>Group</strong></td>
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*The values are given as the mean and the standard error. †The values are given as the mean of the number of vessels in ten high-power fields of view. ‡The values are given as the mean of the number of cells in ten high-power fields of view.
Shoulder stiffness and the severity of shoulder pain were recorded in a standard pain questionnaire format (a modified version of the L’Insalata questionnaire) based on the patient’s self-assessment at his or her first visit. The questionnaire was designed to measure functional capacity in terms of frequency of pain, intensity of pain, and overall shoulder stiffness. Patients responded to Likert-format questions pertaining to the frequency of pain with activity, intensity of night pain, and overall shoulder status. Patient-determined pain severity was ranked as never, sometimes, monthly, weekly, or all of the time. Patients were also asked to state their shoulder stiffness and the difficulty that they had in performing daily tasks such as reaching above the head and reaching behind the back as none, mild, moderate, severe, or very severe.

Patient Demographic Characteristics
Between January 2011 and December 2012, thirty patients who satisfied the inclusion and exclusion criteria were selected for this study. All patients fulfilled the following criteria: a history of shoulder pain and dysfunction, no previous surgical procedure on the affected shoulder, and no radiographic sign of fracture of the shoulder. In addition, those in the calcific tendinitis group had ultrasound and radiographic evidence of calcific tendinitis in the supraspinatus tendon, calcific deposits within the supraspinatus tendon at arthroscopic examination, and no history of rheumatoid arthritis or osteoarthritis. Those in the rotator cuff repair group had evidence of a tear and no rheumatoid arthritis.

These patients were divided into three groups: (1) the calcific tendinitis group, which consisted of ten patients (seven men and three women) with a mean age of fifty-six years (range, forty to sixty-three years); (2) the rotator cuff repair group, which consisted of ten patients (six men and four women) with a mean age of fifty-six years (range, thirty-seven to seventy-seven years); and (3) a control group (treated with stabilization), which consisted of ten patients (nine men and one woman) with a mean age of forty years (range, nineteen to sixty years). The mean reported duration (and standard error) of symptoms was 15.6 ± 3 months in the calcific tendinitis group, 12.0 ± 4 months in the rotator cuff repair group, and 4.8 ± 1 months in the control group (p < 0.05) (Table I).

Imaging
A specialist sonographer (L.H.) with twenty years of experience in musculoskeletal imaging performed the ultrasound imaging on all patients prior to the surgical procedure. In addition, she attended the surgical procedure and used ultrasound to identify and to locate the calcium within the supraspinatus tendon. Ultrasound and radiographs were used to demonstrate calcium deposits within the supraspinatus tendon. Ultrasound imaging was performed with use of an imaging system (LOGIQ E9; GE) with a high-frequency 5 to 16-MHz linear probe, used to identify the calcium within the supraspinatus tendon. The calcium deposit was identified and was measured, and its position with respect to the biceps tendon was noted. The InSight mini C-arm system (Hologic) in the clinic was used to identify the calcium under radiographic imaging.

Tissue Collection
Shoulder arthroscopy was performed with use of the standard three-portal technique as described by Wu et al. A meniscal biter was used to remove a 1 × 1-mm sample adjacent to the calcific material within the supraspinatus tendon, harvesting three to four samples from each patient in the calcific tendinitis group. In the control group, the subscapularis tendon was harvested arthroscopically from the superior border of the tendon approximately 1 cm lateral to the glenoid labrum. In the rotator cuff repair group, supraspinatus tendon was taken from the torn edge of the tendon.

Histology and Immunohistochemistry
Tissue specimens were placed in formalin, were embedded in paraffin, and were stained with hematoxylin and eosin and toluidine blue for determination of the degree of tendinopathy utilizing our previously described modified version of the Bonar score, in which Grade 4 indicated marked tendinopathy, Grade 3 indicated advanced tendinopathy, Grade 2 indicated moderate degeneration, Grade 1 indicated mild degeneration, and Grade 0 indicated a normal tendon.

These were evaluated in a blinded fashion by one of the senior authors (N.L.M.). This included the presence or absence of edema and degeneration together with the degree of fibroblast cellularity and chondroid metaplasia.

The rotator cuff repair group had evidence of a tear and no rheumatoid arthritis. Between January 2011 and December 2012, thirty patients who satisfied the inclusion and exclusion criteria were selected for this study. All patients fulfilled the following criteria: a history of shoulder pain and dysfunction, no previous surgical procedure on the affected shoulder, and no radiographic sign of fracture of the shoulder. In addition, those in the calcific tendinitis group had ultrasound and radiographic evidence of calcific tendinitis in the supraspinatus tendon, calcific deposits within the supraspinatus tendon at arthroscopic examination, and no history of rheumatoid arthritis or osteoarthritis. Those in the rotator cuff repair group had evidence of a tear and no rheumatoid arthritis.

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Thereafter, sections were stained with primary antibodies directed against the following markers: PGP 9.5 for peripheral nerves (polyclonal rabbit anti-PGP 9.5, 1:100 dilution; Invitrogen), GAP-43 for nerves (monoclonal mouse anti-GAP-43, 1:100; Invitrogen), CD34 for vascular endothelium (monoclonal mouse anti-human CD34, 1:800; Dako Australia), CD68 for all macrophages, CD3 for T cells, CD206 for M1 macrophages, and mast cell tryptase for mast cells. Endogenous peroxidase activity was quenched with 3% (vol/vol) H2O2, and nonspecific antibody binding was blocked with 2.5% horse serum in a Tris-buffered saline and Tween 20 (TBST) buffer for thirty minutes. Antigen retrieval was performed in 0.01-M citrate buffer for twenty minutes in a microwave. Sections were incubated with primary antibody in 2.5% (wt/vol) horse serum, human serum, and TBST at 4°C overnight. After two washes, slides were incubated with the ImmPRESS reagent kit (Vector Laboratories) as per the manufacturer’s instructions for thirty minutes. The slides were washed and were incubated with the ImmPACT DAB chromogen solution (Vector Laboratories) for two minutes, followed by extensive washing. Finally, the sections were counterstained with hematoxylin. These methods have been described previously. Positive control specimens for PGP 9.5 and GAP-34 (normal skin, as it reliably contains myelinated and unmymelinated nerve fibers expressing these) and for CD34 (CD34-positive endothelial cells) as well as negative control specimens for these three markers were included, in addition to the surgical specimens for each individual antibody staining technique. The omission of primary antibody and use of negative control isotypes confirmed the specificity of staining.

We applied a scoring system based on our previous experience to quantify the immunohistochemical staining. Ten random high-power fields (×400) were evaluated by two independent blinded assessors (N.L.M. and G.A.C.M.). In each field, the numbers of positive and negatively stained cells were counted, the percentage of positive cells was calculated, and the mean percentage was calculated from the values of the two reviewers, giving the following semiquantitative grading: Grade 0 indicated that there was no staining, Grade 1 indicated that <10% of cells stained positive, Grade 2 indicated that 10% to 20% of cells stained positive, and Grade 3 indicated that >20% of cells stained positive. In addition, the blood vessel numbers were assessed in the same random high-power fields. Intrarater reliability was good, reflected by the r value of 0.84.

Statistical Analysis
Results are reported as the mean value and the standard error. Comparisons between groups were made with two-way paired Student t tests, Mann-Whitney U tests, and Kruskal-Wallis one-way analysis of variance on ranks. Based on our previous immunohistochemical studies and power calculations (power of 0.8 and beta error of 0.2), we identified that each group required ten patient samples to detect a 20% difference in immunostaining for the various immune, nerve, or vascular markers. Correlations were calculated with use of the Pearson coefficient.

Source of Funding
There was no external funding source for this study.

Results
Increased Neovascularization and Neoinnervation in Calcific Tendinitis
Patients with calcific tendinitis showed a significantly greater vessel count compared with the rotator cuff repair group and control group (p < 0.001). Patients in the rotator cuff repair group also had a significantly greater vessel count compared with the control group (p < 0.001) (Figs. 1 and 2, Table II). Additionally, patients with calcific tendinitis had significantly greater neoinnervation (p < 0.001) compared with both
Fig. 2
Immunostaining for neovascularization (CD34) and peripheral nerve (PGP 9.5) markers in calcific compared with control tendon. Blue arrows denote staining of endothelial cells around new vessels in the calcific tendon. Black arrows denote positive staining of peripheral nerves in the calcific tendon.

Fig. 1
Figs. 1-A through 1-D Blood vessel and nerve counts. Figs. 1-A and 1-B The mean value (and standard error) for the vessel count and nerve count per high-power field (HPF) in patients with calcific tendinitis compared with patients with a rotator cuff tear and controls. There were ten patients in each group. Significance was denoted by three asterisks for p < 0.001 and two asterisks for p < 0.01. The error bars indicate the standard error. Figs. 1-C and 1-D Blood vessel count per HPF correlated with patient-reported frequency of pain during sleep and frequency of extreme pain, as shown by the indicated Pearson coefficient.
Inflammatory Cell Infiltrate in Calcific Tendinitis

Macrophages

CD68-positive cells (i.e., macrophages) and M₂ macrophages were both significantly more common (p < 0.001) in the calcific tendinitis group compared with the rotator cuff repair group and the control group (Figs. 3-A and 3-B, Table II). The increased presence of CD68-positive cells (r = 0.8, p < 0.01) and M₂ macrophages (r = 0.6, p < 0.01) positively correlated with the nerve count. Similarly, the increased presence of CD68-positive cells (r = 0.8, p < 0.01) and M₂ macrophages (r = 0.8, p < 0.01) positively correlated with increased neovascularization (Fig. 3).

Mast Cells

There were significantly greater mast cell counts in the calcific tendinitis group compared with the rotator cuff repair group and the control group (p < 0.001) (Fig. 3-D). The rotator cuff repair group also had significantly greater mast cell counts compared with the control group (p < 0.01). Increased mast cells positively correlated with an increase in neoinnervation (r = 0.7, p < 0.01) (Fig. 3-D).

Fig. 3

Figs. 3-A through 3-D Macrophages and mast cells. There were ten patients in each group. Significance was denoted by three asterisks for p < 0.001 and two asterisks for p < 0.01. The error bars indicate the standard error. Figs. 3-A and 3-B The mean number (and standard error) of total (CD68-positive) and M₂ macrophages per high-power field (HPF) in patients with calcific tendinitis compared with patients with a rotator cuff tear and controls. Fig. 3-C Correlation of M₂ (top) and total (bottom) macrophage counts with neoinnervation and neovascularization, respectively (with Pearson coefficients). Fig. 3-D Mast cells per HPF in patients with calcific tendinitis compared with patients with a rotator cuff tear and controls (top), and correlation of mast cell counts with neoinnervation (with Pearson coefficients) (bottom).
T Cells

There were no significant differences (p > 0.05) in T-cell count between the calcific tendinitis group and the rotator cuff tear group; however, both the calcific tendinitis group and the rotator cuff repair group had fivefold to sixfold higher T-cell counts compared with the control group (p < 0.001). Increased T cells positively correlated with nerve count (r = 0.4, p < 0.05) and neovascularization (r = 0.6, p < 0.01).

Clinical Outcomes

There was a significant difference (p < 0.05) in greater pain intensity in the calcific tendinitis group, and these patients experienced extreme pain more frequently than the rotator cuff repair group and the control group (p < 0.05). In addition, the patients with calcific tendinitis had less passive abduction (p < 0.01), forward flexion (p < 0.05), internal rotation (p < 0.01), and external rotation (p < 0.01) than the control group.

Discussion

This investigation showed a significantly elevated amount of neovascularization and neoinnervation in calcific tendinitis lesions of the shoulder. The calcific tendinitis group had significantly greater nerve counts compared with the rotator cuff repair group and the control group. A greater nerve count positively correlated with a greater degree of neovascularization and also positively correlated with greater frequency of extreme pain. Neovascularization also positively correlated with more frequent pain during sleep and more frequent extreme pain.

The pathophysiology of calcific tendinitis remains largely unknown, with proposed theories including degenerative calcification11, repetitive trauma22, necrosis of tenocytes and intracellular calcium accumulation25, reactive calcification1, endochondral ossification14, and chondral metaplasia13. Although it is one of the most painful shoulder pathological conditions, to our knowledge, few molecular works have examined the role of the peripheral neuro-phenotype in these patients. Our findings are consistent with previous studies relating to increased neovascularization and neoinnervation causing pain in patients presenting with frozen shoulder, rotator cuff tears26,18, and other musculoskeletal pathological conditions. In our patient subsets, there was a twofold to eightfold increase of nerve markers, neovascularization, macrophages, M2 macrophages, and other inflammatory mediators and peripheral nervous and vascular cells in contributing to the pathogenesis and likely the pain associated with calcific lesions. Indeed, previous investigations of tendinopathy also demonstrated a modest twofold increase in neovascularization or neoinnervation compared with our twofold to eightfold increase, suggesting that the increased pain associated with calcific tendinitis may be linked to an increased peripheral nerve and vascular phenotype.

Dysregulated tissue repair and inflammation characterize many common musculoskeletal pathological conditions, including tendon disorders26. Increasing evidence points toward an early inflammatory infiltrate and associated inflammatory cytokine production in human and animal models of tendon disease27. To our knowledge, few works have investigated the presence and/or role of immune cells and their molecular messengers (cytokines or chemokines) in calcific tendinitis. Herein, we identify that innate immune cells (macrophages or mast cells) represent an important cellular subtype within calcific tendon compared with both torn and normal tendons, suggesting that inflammation plays a critical role in calcific tendinopathy.

The main limitation of this study was the relatively small sample size of the cohorts. Additionally, there was selection bias in that we only studied patients with calcific tendinopathy that warranted a surgical procedure and thus did not include asymptomatic patients or patients with less pain intensity with calcium deposits, which may represent an altered pathophysiology. Our main strength was the use of a control group of patients without calcific tendinitis or rotator cuff tear to serve as a comparison. Additionally, we utilized well-described and reproducible staining techniques and sampling of tissue performed by the same surgeon (G.A.C.M.).

In summary, our cellular and molecular findings of inflammation, neoinnervation, and neovascularization in calcific tendon may suggest a pathophysiological mechanism for the pain associated with calcific tendinitis.

References


2. Ruttimann G. Huber die Häufigkeit rontgenologischer Veränderungen bei Patienten mit typischer Periarthritis humeroscapularis und Schultergesunden


