Correlations among mineral components, progressive calcification process and clinical symptoms of calcific tendonitis

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Abstract

Objective. To establish the correlations among the mineral components, progressive calcification process and clinical symptoms of calcific tendonitis.

Methods. The morphology of the calcified deposits on the shoulders of 28 patients with calcific tendonitis was determined by high-resolution ultrasonography. The calcified deposit from each patient was aspirated and determined by the Fourier transform infrared and Raman microspectroscopies. The curve-fitting program was applied to estimate the chemical component in the calcified deposits of calcific tendonitis.

Results. The morphology of calcified deposits for 28 patients was classified into four shapes: arc shape (7 patients), fragmented/punctuate shape (4 patients), nodular shape (13 patients) and cystic shape (4 patients). These classified shapes markedly correlated with the pain levels in patients. The infrared spectra of all the calcified deposits for 28 patients were easily classified into three types in the blind study and corresponded to the formative, resting and resorptive phases in the progressive calcification process of calcific tendonitis. With the progressive calcification, the IR wavenumber at 1018 cm\(^{-1}\)/C\(_{0}\) assigned to poorly crystalline, non-stoichiometric apatite for the formative phase was shifted to 1028 cm\(^{-1}\) for the resting phase and then to 1031 cm\(^{-1}\) due to matured crystalline stoichiometric apatite for the resorptive phase. The curve-fitted results revealed that calcified deposits in calcific tendonitis were composed of different quantities of A-type and B-type carbonated apatites in the three phases. A significant difference was found in carbonated apatite content among the three phases (\(P < 0.001\)).

Conclusions. The different quantities of A-type and B-type carbonated apatites determined by vibrational microspectroscopy in calcified deposits were well correlated with those of the four shapes of morphologic classification, with the three phases in the progressive calcification process and with the clinical symptoms of calcific tendonitis.

Key words: Calcified deposits, Calcific tendonitis, Spectral biodiagnosis, Morphology, Fourier transform infrared spectroscopy, Raman, A-type and B-type carbonated apatites, Progressive calcification, Clinical symptoms.

Introduction

Calcific tendonitis of the shoulder is due to calcified deposits located within the tendons of the rotator cuff causing inflammation and painful symptoms [1, 2]. Although it is difficult to verify whether the pain is attributed to the tendonitis or the calcified deposits themselves, the acute pain may occur when the calcified deposits are undergoing resorption via phagocytosis [1, 3]. Until today, the aetiology and pathogenesis of calcific tendonitis remained unclear, but the calcification following metaplasia in the tendon is widely recognized [4, 5].
Uthhoff et al. [4, 6] had proposed a cyclically evolutionary calcification process for calcific tendonitis: pre-calcification stage → calcification stage → post-calcification stage. The second calcification stage in this cyclic process might also be subdivided into formative, resting and resorption phases. These three phases were induced by the active mediation of calcification due to cells in a viable condition, and caused shoulder pain [6–8]. According to the time-dependent pain symptom and time-associated calcification progression, we have proposed and reconstructed an evolutionary model of the progressive calcification process and clinical symptoms of calcific tendonitis of the shoulder, as shown in Fig. 1 [4, 9]. The diagnosis of this disease is based on clinical examination by using a variety of imaging techniques [9–11]; however, it may be markedly influenced by the experience of the radiologist and instruments used. The existing classification and diagnosis based on radiographs showed different consistency and reliability. It will be interesting to see whether there is a correlation between the extent of calcification and clinical symptoms in these three phases.

It has been reported that chronic or acute calcific tendonitis was predominantly caused by the deposition of hydroxyapatite (HAP) in the periaricular tendon, but no significant differences were found in the symptoms of either type [8]. An inflammatory reaction might be triggered by these HAP crystals through the up-regulation of inflammation mediators. The inflammatory potential of HAP crystals has been reported to vary according to crystal features, such as specific surface area, size and density of the crystals, as well as the calcium/phosphate ratio. The physicochemical heterogeneity of crystals might also be an additional factor leading to inflammation [12, 13]. The carbonate ion (\(\text{CO}_3^{2-}\)) is known to occupy two different positions in the crystal lattice of HAP $[\text{Ca}_{10}(\text{PO}_4)_{6}(\text{OH})_2]$; it can substitute for the \(\text{OH}^-\) site (defined as A-type carbonated apatite) or for the $\text{PO}_4^{3-}$ site (defined as B-type carbonated apatite), in which the B-type carbonated apatite is the major mineral species of the biological apatites [14, 15]. Whether the crystal features of the carbonated apatite may affect the inflammatory properties and clinical symptoms is unknown. Although the carbonated apatite has been reported to be a single component in the calcified deposits of calcific tendonitis [2, 8, 16], there are no investigations concerning the role of the types of carbonated apatites in calcific tendonitis.

Recently, the use of vibrational spectroscopy has shown great potential over other diagnostic techniques to successfully investigate the chemical composition of the diseased tissue, rather than histological pathology alone [17, 18]. The Fourier transform infrared (FT-IR) and Raman spectroscopies provide similar but complementary information on molecular-structural fingerprints of the samples. Both techniques are suitable to analyse the mineral structure with fast and non-destructive analysis, since they are also sensitive to the changes in crystallinity or molecular substitution. They can directly determine the tiny samples via the microscope system without special sample preparation. In our previous studies, several human calcified tissues, such as calcinosis cutis, skin pilomatrixoma, cornea, senile cataractous lens, vitreous asteroid bodies and sclera, had been investigated by using these vibrational microspectroscopic techniques [18, 19]. The purpose of this study was to quantitatively determine the type and content of chemical components in the calcified deposits of calcific tendonitis by FT-IR and Raman microspectroscopies. Moreover, the correlations of the mineral composition in calcified deposits with progressive calcification process or with clinical courses and symptoms of calcific tendonitis were also explored.

**Patients and methods**

**Patients**

Twenty-eight patients with shoulder pain due to calcific tendonitis were enrolled in this study [11 men and 17 women; age range 46–81 (average 57) years]. All patients were referred from the outpatient department of Orthopaedics. The enrolled criteria in patients included chronic shoulder pain for at least 6 months with or without acute exacerbation. Diagnostic high-resolution ultrasonography (HRUS) was carried out initially to verify the calcific tendonitis. The HRUS with a GE Voluson E8 system (GE Medical Systems, Milwaukee, WI, USA) using S10-16-D
linear probe and Philips IU22 system, and HDI 5000 using L12-5 linear probe (Philips-ATL, Bothell, WA, USA) were used for imaging measurements. Each patient was explained the procedure very clearly and signed the consent form before the procedure. The present study was approved by the Institutional Review Board at the Taipei Veterans General Hospital according to the Declaration of Helsinki.

**Clinical evaluation method**

All patients had undergone an HRUS examination and showed a calcific tendonitis. The clinical symptoms were modified from the visual analogue scale (VAS) system, and patient symptoms were graded as painless (0), mild (1–4), moderate (4–8) and severe (8–10), according to the patient’s chief complaint and the compression of a probe to the calcified deposits [5, 20, 21]. There were 10 patients with mild shoulder pain, 8 patients with moderate-degree shoulder pain and 10 patients with severe shoulder pain in this study. The morphology of the calcified deposit of the shoulder determined by HRUS was classified into four shapes: arc (an echogenic arc with clear shadowing, Fig. 2A); fragmented or punctuate (at least two separated echogenic spots or plaques with or without shadowing, Fig. 2B); nodular (an echogenic nodule without shadowing, Fig. 2C); and cystic (a bold echogenic wall with an anechoic area, weak internal echoes or layering content, Fig. 2D).

**Aspiration method**

The ultrasound-guided puncture was repeatedly undertaken for each patient, according to our previous method [5, 20, 21]. In the puncture procedure, which was performed with the free-hand method monitored with HRUS, the needle tip was placed in the calcified plaque, which was then punctured by moving the needle back and forth with 2-ml negative pressure aspirated. After the procedure, the puncture site was bandaged with hand compression for 15 min by the patient himself who was then sent back home without any complications. The calcified deposit was aspirated into two parts for histopathological examination and vibrational microspectroscopic study.

**Histopathological examination**

Each calcified deposit was processed routinely to produce 5-μm paraffin-embedded tissue sections and stained with haematoxylin and eosin (H&E). The sample

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**Fig. 2** The representative sonogram of each calcified deposit of the shoulder examined by HRUS.

(A) An arc-shape calcification (arrows) was found over the supraspinatus tendon in a 48-year-old female who complained of right-shoulder pain for >6 months. (B) A punctuate calcification (arrows) was observed in the right supraspinatus tendon in a 46-year-old female who complained of right-shoulder pain for 1 year. (C) A fragmented nodular type calcification (arrows) in right shoulder was found in a 53-year-old female patient who complained of chronic shoulder pain for >1 year. (D) A cystic type (arrows) with bold echogenic rim on the left subscapularis tendon was observed in a 53-year-old female patient who complained of chronic pain over the left shoulder for >2 years.
was observed by using the light microscopy (BH-2, Olympus, Tokyo, Japan).

Vibrational microspectroscopic study
The blind study was initially performed and the code name was only given for spectral analysis. The sample of calcified deposit was washed with distilled water and centrifuged. The process was carried out twice. The sample was then dried for 1 day at 25°C, 50% relative humidity conditions. The dried samples were directly examined to identify and determine the chemical component by using both FT-IR microspectroscopy (Micro FTIR-200; Jasco Co, Tokyo, Japan) with a transmission technique and confocal micro-Raman spectrophotometer (Jasco Ventuno, Jasco Co., Tokyo, Japan) equipped with a 30-mW green (532 nm) solid-state laser standard via non-destructive analysis [18, 19]. The IR spectra were carried out at 200 scans and a resolution of 4/cm, but the pixel resolution of Raman system was 1.3/cm. Synthetic HAP (>100 mesh; purity, >99.5%; Nacalai Tesque, Tokyo, Japan) was used as a standard reference.

Spectral data acquisition and handling
(1) Spectral analysis. A spectra manager (Jasco Co., Tokyo, Japan) and GRAMS spectroscopy software suite (Thermo Electron Co., Waltham, MA, USA) were used for spectral data processing. Second-derivative FT-IR spectral analysis was applied to locate the position of the overlapping components of samples.

(2) Compositional component determined by curve-fitting program. The IR spectral ranges within 888 and 862/cm were quantitatively estimated by the curve-fitting algorithm using the Gaussian function with the minimum standard error. The composition of each component was computed to be the fractional area of the corresponding peak, divided by the sum of the areas of all the peaks.

(3) Statistical analysis. For comparisons of these variables, analysis of variance (ANOVA) was used. All results were expressed as the mean (S.D.). P-values <0.05 were considered to be of significant difference.

Results
The histopathological examination of the sliced sample revealed a deep blue–purple on the H&E stain, implying the existence of a calcified deposition [22]. Figure 2 shows the representative sonogram of each calcified deposit of the shoulder examined by HRUS, in which the arrows indicate the shape of calcification. It clearly indicates that the HRUS could easily classify the calcified deposits into four shapes including the arc (Fig. 2A), fragmented/punctuate (Fig. 2B), nodular (Fig. 2C) and cystic (Fig. 2D). In the present study, the morphology of the calcified deposits may be classified into an arc shape in 7 patients with clinical symptoms (6 mild and 1 moderate), fragmented/punctuate shape in 4 patients (3 mild and 1 severe), nodular shape in 13 patients (1 mild, 6 moderate and 6 severe) and cystic shape in 4 patients (1 moderate and 3 severe), as shown in Table 1. That is, there were 16 out of 18 patients with moderate to severe clinical symptoms occurring in nodular or cystic type calcification. Obviously, there was a positive correlation between the morphology of the calcified deposits and the clinical symptoms of the shoulder pain with significant difference.

All the infrared (IR) spectra of calcified deposits for the 28 patients with calcific tendonitis can be clearly classified into three types in the blind study, as shown in Fig. 3A-a, b and c. The IR spectrum of HAP reference standard is also displayed (Fig. 3A-d). It is surprising to note that each type of IR spectrum was exactly attributed to that of the above morphologic classification. The representative IR spectrum in Fig. 3A-a might refer to the IR spectrum of calcified deposits for the arc and fragmented/punctuate types. The representative IR spectrum in Fig. 3A-b or -c belonged to the IR spectrum of calcified deposits for the nodular or cystic type, respectively. According to the time-dependent fate of calcified deposits in Fig. 1, these three types of IR spectra may be defined as the results of formative, resting and resorptive phases in the calcification stage of progressive calcification process of calcific tendonitis (Table 1).

These three types of IR spectra were also closely similar to each other in many positions of the wavenumber, except the predominant IR peaks ≈1018–1031/cm assigned to the \(\nu_2\) stretching vibration of phosphate for the apatite structure [17, 18, 23]. The IR peaks at 872–875, 1413–1416 and 1451–1455/cm were due to the IR wavenumbers of \(\nu_2\) stretching mode of carbonate bands in these calcified samples, but the IR peak at 959–961/cm corresponded to the \(\nu_1\) symmetric stretching phosphate. The IR spectrum of HAP indicates several characteristic

<table>
<thead>
<tr>
<th>Morphologic shape</th>
<th>Patient no.</th>
<th>Painless</th>
<th>Mild</th>
<th>Moderate</th>
<th>Severe</th>
<th>Calcific stage proposed</th>
</tr>
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<tr>
<td>Arc</td>
<td>7</td>
<td>0</td>
<td>1</td>
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<td>0</td>
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</tr>
<tr>
<td>Fragmented/punctuate</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Formative phase</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Resting phase</td>
</tr>
<tr>
<td>Cystic</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>Resorptive phase</td>
</tr>
</tbody>
</table>

Table 1 Correlations among the shapes of calcified deposits, clinical symptoms and calcification stages proposed
absorption peaks at 875, 961, 1031, 1090 and 3569/cm, in which the peaks at 961, 1031 and 1090/cm corresponded to the stretching modes of phosphate but at 875/cm attributed to carbonate ions. A unique peak positioned at 3569/cm was due to the OH-group of HAP. In the fingerprint region of 900–1200/cm, a predominant peak at 1018/cm for calcified deposits of formative phase was gradually shifted to 1031/cm with the progressive calcification process and it was close to that of the IR spectrum of HAP. This clearly indicates that apatite was a key component in the calcified deposits of calcific tendonitis. Moreover, the amide I and II bands of protein at 1643–1654 and 1541–1545/cm were also observed in the IR spectra of these calcified deposits. The 2800–3000/cm spectral region attributed to the CH₂ and CH₃ stretching vibrations of protein was also found. This reveals that some proteins had adhered to the calcified sample [17, 18, 23].

On the other hand, there was no significant difference among Raman spectra in the three phases, as displayed in Fig. 3B. Several small peaks at 2800–3000, 1665–1667 and 1449–1452/cm corresponding to the protein structure and a peak at 1000–1004/cm assigned to HPO₄²⁻ and/or phenylalanine were obtained in the Raman spectra of the three phases. A predominant peak at 958–961/cm and a small peak at 1071–1072/cm were found in these Raman spectra.
spectra, which were due to the $\nu_1$ stretching mode of phosphate and carbonate, respectively [17, 18, 24]. The typical Raman spectrum of HAP was normally found at 961 and 1045/cm due to $\nu_1$ and $\nu_3$ stretching vibration of phosphate. A unique peak at 3576/cm due to the OH group was also observed in the Raman spectrum of HAP. The OH band at 3569/cm in IR spectrum and at 3576/cm in Raman spectrum was absent from all the IR and Raman spectra, which implies complete occupation of the OH sites of HAP by carbonate in all calcified samples [8].

Discussion

It is well known that crystal deposition is also a type of biomineralization process causing many disorders and symptoms in different human organs [25, 26]. Although calcific tendonitis of the rotator cuff is one of the crystal deposition disorders and is also characterized by an inflammation around the HAP crystal deposits, the progressive calcification process and clinical symptoms of calcific tendonitis remain unclear. In this study, the morphology of calcified deposits was variable from arc type to cystic type. Table 1 indicates that with the exacerbation of calcification from arc to cystic, the clinical symptoms were more serious, from mild to severe, suggesting that the morphology of calcified deposits was well correlated with the clinical symptoms of calcific tendonitis. The result was similar to our previous study [5, 20, 21]. Furthermore, the changes in morphology were also correlated with the fate of calcified deposits from mineralization, enlargement and liquefaction, and phagocytosis by accompanying clinical symptoms of shoulder pain (Fig. 1).

According to the histology, there are three recognized stages of calcific tendonitis including the pre-calcification, calcification and post-calcification stages [4, 7]. Since most patients are asymptomatic during the pre-calcification phase, the calcification stage subdivided into the formative, resting and resorptive phases should be the main clinical symptom on which to base a diagnosis and give treatment in hospital. However, the intact constitution of calcified deposits in each type is unknown. Understanding the exact composition of calcified deposition at each phase and stage in calcific tendonitis is of great importance in a variety of pathological diagnosis.

In the spectral analysis of the mineralization process, two IR spectral regions at 900–1200/cm ($\nu_1$ and $\nu_3$ stretching modes of phosphates) and 860–890/cm ($\nu_2$ stretching mode of carbonate) of mineralized materials had recently been used for FT-IR spectroscopic examination [17, 18, 27]. It has been reported that a peak near 1020/cm is attributed to the $\nu_3$ vibration of $\text{PO}_3^{4-}$ in poorly crystalline, non-stoichiometric apatites, but a peak at 1030/cm is indicative of the $\nu_3$ vibration of $\text{PO}_3^{4-}$ in matured crystalline stoichiometric apatites [27, 28]. Thus, the crystallinity and maturity of biological apatites can be estimated by the peak intensity or peak area ratio of 1020/1030/cm. With maturation, apatitic lattice may evolve towards a greater order leading to the decrease in the ratio value of 1020/1030/cm. Here, the HAP sample used in this study exhibited a unique peak at 1031/cm, indicating that it belonged to a matured crystalline stoichiometric apatite.

As shown in Fig. 3A, a sample of the formative phase showed a predominant IR peak at 1018/cm, suggesting that the calcified deposits of calcific tendonitis at this phase consisted of the poorly crystalline, non-stoichiometric apatites [27, 28]. With the progressive calcification process of calcific tendonitis, the wavenumber of this unique peak shifted from 1018/cm (formative phase) to 1028/cm (resting phase) and then to 1031/cm (resorptive phase). The calcified deposits of the resorptive phase seemed to be close to the matured crystalline stoichiometric apatite of HAP used. The maturation of apatite from the poorly crystalline, non-stoichiometric state at the early stage to the matured crystalline stoichiometric state at the later stage was associated with the progressive calcification of calcific tendonitis, indicating that mineralization progressively occurred in the calcified deposits of calcific tendonitis.

FT-IR vibrational spectroscopy has also been applied to determine the location of the carbonate ions in biological minerals [14, 15, 17, 18]. Carbonate is one of the substituting ions; it may substitute for either the OH$^-$ (A-type carbonated apatite) or $\text{PO}_4^{3-}$ (B-type carbonated apatite) anions in the crystal lattice of apatites or it may be in unstable locations. IR spectroscopic results of the fully mineralized samples have shown that the carbonate bands in the $\nu_2$ $\text{CO}_3^{2-}$ domain region have three components: the major component near 871/cm is due to the B-type carbonate; a band near 878/cm is assigned to the A-type carbonate; and a band ~866/cm is due to the liable carbonate ions located in the poorly crystalline environments and not incorporated into the apatitic lattice [14, 15]. With the increase of maturation, the 866/cm shoulder intensity decreases as compared with the 871/cm intensity.

In this study, the typical spectral region of $\nu_2$ stretching mode of $\text{CO}_3^{2-}$ and the three underlying bands are deduced by a curved-fitting analysis, as shown in Fig. 4. Obviously, three components were included in the spectral region of 862–888/cm. The assignment and composition of the curve-fitted original IR spectrum of HAP were obtained as follows: 882/cm (15.1%), 875/cm (65.1%) and 868/cm (19.8%). Three components of each calcified deposit for 28 patients are revealed in Fig. 5. All data were close to each other and the differences in variability between the same groups were minimized. The mean (S.D.) values of each component in different phases are also shown in Fig. 5. Statistical analysis indicates that significant differences were observed among the three phases: formative phase and resting phase ($P < 0.001$); formative phase and resorptive phase ($P < 0.01$–0.001); resting phase and resorptive phase ($P < 0.001$) except the liable carbonate ions ($P > 0.05$). It strongly indicates that the IR spectral region of 862–888/cm for carbonate was a useful range for differentiating the progressive calcification process. Moreover, the classification of three phases in the progressive calcification process of calcific tendonitis was validated.
Since the composition of the 866/cm band was reduced with the progressive calcification process, the mineralization in calcified deposits of calcific tendonitis has gradually grown towards the maturation. It clearly reveals that carbonate ions located in the liable sites were lost during the maturation process. Moreover, a slight decrease of those located in the A-type position and an increase of those located in the B-type site is also indicated in Fig. 5. The result strongly supports that the maturation of mineral fractions in calcified tissues of calcific tendonitis also gradually proceeds in the progressive calcification process.

There are three limitations in the present study. First, the specimens of calcified deposits were sampled from the rotator cuff. Secondly, the cases of resorptive phase in the progressive calcification process were less than other phases, since some calcified deposits were gradually resorbed into the body leading to the lack of sampling. Thirdly, the change in the composition of the calcified deposits in the same patient was not available in the present study because the patient refused the puncture again in a short time. Further studies are needed to investigate the composition of calcified deposit in ineffective repeated punctures of calcific tendonitis.

In conclusion, the result of the present study indicates that different quantities of A- and B-type carbonated apatites in calcified deposits are reflected in the formative, resting and resorption phases in the calcification stage of the progressive calcification process of calcific tendinitis. Reduced amounts of carbonates located in the liable and in A positions but increased amounts located in B positions were found in this progressive calcification process. Moreover, these three phases in the calcification stage of the progressive calcification process were well correlated with the morphology of calcified deposits and clinical symptoms of shoulder pain for calcific tendonitis. Correlations among the mineral components, progressive calcification process and clinical symptoms of calcific tendonitis were established.

**Rheumatology key messages**

- Vibrational spectroscopy effectively evaluated the types and contents of carbonated apatites in the calcified deposits.
- Carbonated apatites located in the liable and A positions were reduced but increased in the B positions.
- Compositional changes in the calcified deposits were well correlated with morphology, progressive calcification process and clinical symptoms.

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