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ORIGINAL ARTICLE

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Localization and expression of osteopontin in the rotator cuff tendons in patients with calcifying tendinitis

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Abstract Calcifying tendinitis of rotator cuff tendons is a common and painful condition caused by ectopic calcification in humans. To examine the involvement of osteopontin (OPN), a potent regulator of calcium deposition on connective tissues, localization and expression of OPN protein and messenger (m)RNA were investigated in human tissue samples of calcified rotator cuff tendons. Immunohistochemistry demonstrated that OPN was localized in cells surrounding the calcified area. OPN was localized in two distinct cell types, i.e., fibroblast-like cells negative for CD68 and tartrate-resistant acid phosphatase (TRAP) and multinucleated macrophages positive for CD68 and TRAP. In situ hybridization revealed that the mRNA expression of OPN in these cells coincided with the immunohistochemistry results, and these results were supported by reverse transcriptase polymerase chain reaction analysis using human OPN-specific oligonucleotides. Cells located away from the calcified area did not express OPN. The present findings indicate the involvement of OPN in the process of calcification of rotator cuff tendons and suggest that OPN plays a role in such painful disorders through the actions of at least two cell types.

Keywords Osteopontin · Tendon · Calcification · In situ hybridization · Immunohistochemistry

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Introduction

Calcification at an extraskeletal site is associated with a significant number and variety of disorders in humans [36]. In particular, ectopic calcification occurring in connective tissues at peri- or intra-articular sites, i.e., tendon or synovium, sometimes leads to very painful conditions. Thus, pathomechanisms regarding the process of ectopic calcification at peri- or intra-articular sites need to be elucidated. However, little is known about the cellular mechanisms or local factors involved in this condition [36].

Among various kinds of ectopic calcification at peri- or intra-articular sites, calcifying tendinitis of the shoulder joint is the most common and painful disease, involving the deposition of calcium salts in the rotator cuff tendons [7, 31]. Diagnosis of this disease is based on radiological findings showing a characteristic shadow of calcification (Fig. 1) [14]. Although ultrastructural and biochemical studies have clarified the pathogenesis of calcifying tendinitis [1, 9, 24, 34], the molecules and cells involved have yet to be examined.

Osteopontin (OPN) is one of the major non-collagenous bone matrix proteins that is associated with bone formation and mineralization [6]. OPN has been reported to be synthesized by the cells in mineralizing developmental tissues [13, 25, 26], and several biochemical studies suggest that OPN regulates cell attachment, migration [22, 23], and tissue mineralization [4]. In addition, OPN has been reported to play an important role in the calcification that occurs in several pathological conditions in humans, including arteriosclerosis [8, 10, 12, 18, 27, 28, 32] and urinary stones [2, 15, 20, 21, 33]. However, no reports exist regarding the involvement of OPN in ectopic calcification in tendon tissues.

The aim of the present study was to examine whether OPN is involved in the process of calcification in the rotator cuff tendons of calcifying tendinitis utilizing human surgical samples. Cellular localization of the OPN gene and protein was determined using in situ hybridization

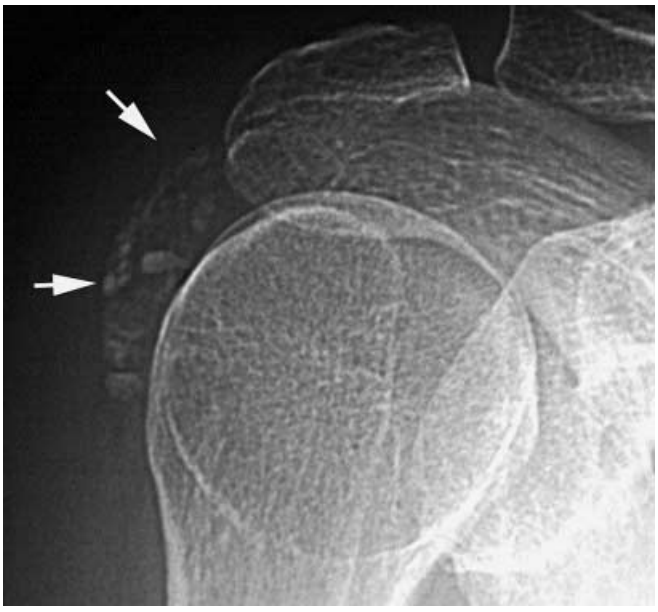

Fig. 1 Representative radiograph showing calcium deposition (*arrows*) in a patient with calcifying tendinitis of the rotator cuff tendon

and immunohistochemistry, respectively. Expression of OPN messenger (m)RNA was confirmed using reverse transcription polymerase chain reaction (RT-PCR) analysis.

Patients and methods

Patients

Rotator cuff tendon tissue containing a calcified area was obtained during surgery from five patients with calcifying tendinitis in whom conservative treatment was not effective. A representative X-ray of one patient is shown in Fig. 1. As controls, non-calcified rotator cuff tendon tissues were obtained from two patients with rotator cuff tears. Patients' details are summarized in Table 1. Informed, written consent was obtained from all patients.

Preparation of tissue sections

Tissue samples were prepared as previously described [19] with modifications. They were fixed in 4% paraformaldehyde (Merck KGaA, Darmstadt, Germany) in phosphate-buffered saline (PBS; pH 7.4; Sigma Chemical Co., St. Louis, Mo.), and then dehydrated through an ethanol series and embedded in paraffin. Sections 5-µm thick were cut using a microtome, stained with hematoxylin and eosin and then with von Kossa's method to reveal calcium deposits. Remaining serial sections were prepared for immunohistochemistry and in situ hybridization.

Antibodies

Mouse monoclonal antibodies against human OPN [11, 30] and human CD68 (KP-1) was purchased from American Research Product Inc.(Mass.) and Dako (Calif.), respectively. Anti-human OPN antibody was diluted to 10 µg/ml in PBS and anti-human CD68 antibody was diluted 1/50 in PBS. They were used as primary antibodies in immunohistochemistry as described hereafter.

Immunohistochemistry

Immunohistochemistry was performed using the streptavidin-peroxidase method with histofine SAB-PO kits (Nichirei, Tokyo, Japan) according to the method recommended by the manufacturer [29]. Tissue sections were briefly deparaffinized and dehydrated and placed in 3% H_2O_2 in methanol to block endogenous peroxidase. After washing in PBS (pH 7.2), the sections were blocked with 10% normal serum of the same species as the secondary antibody to minimize background staining, followed by incubation with the primary antibody for 2 h at room temperature. Normal serum of the same species as the primary antibody was used as a control for the primary antibody. After washing in PBS, the sections were incubated with secondary antibody [mouse immunoglobulin (Ig)-G: Nichirei] for 20 min at room temperature in a humid chamber and then incubated with peroxidase-conjugated streptavidin (Nichirei) for 20 min at room temperature in a humid chamber and washed in PBS. Finally, the color reaction was performed using the substrate reagent 3,3′diaminobenzidine tetrahydrochloride (Dojindo, Tokyo, Japan). Sections were counterstained with hematoxylin and mounted. TRAP staining was performed using a TRAP staining kit (Sigma). TRAP activity was detected according to the procedure with naphthol AS-TR phosphate containing 10 mM L(+)–tartaric acid as a substrate. These sections were also counterstained with hematoxylin.

In situ hybridization

For generation of the human OPN probe, a 0.54-kb fragment of human OPN complimentary (c)DNA [37] (646–1188) was prepared and subcloned into the pGEM-T plasmid. The plasmid was either linearized with *Sac*II and transcribed by SP6 RNA polymerase to generate an antisense probe or linearized with *Spe*I and transcribed by T7 to generate a sense probe. In situ hybridization was performed as described previously [19, 25]. Briefly, paraffin sections were dewaxed and fixed with 4% paraformaldehyde in 0.1 M PBS. They were then treated with 0.2 N HCl for inactivation of endogenous alkaline phosphatase and acetylated with

Table 1 Summary of the patients with calcifying tendinitis. All patients were Japanese. *Calc. T.* calcifying tendinitis

Case	Age at surgery (years)	Gender	Affected side	Period of duration of pain	Diagnosis
1	55	Female	Left	9 Months	Calc. T.
2	60	Female	Right	3 Years	Calc. T.
3	46	Female	Right	3 Years	Calc. T.
4	63	Female	Right	6 Months	Calc. T.
5	63	Female	Right	2 Years	Calc. T.
6	64	Female	Right		Cuff tear (control)
7	66	Female	Left		Cuff tear (control)

0.25% acetic anhydride in 0.1 M triethanolamine (pH 8.0). This was followed by dehydration with an ethanol series and air drying. A hybridization solution (50 µl) containing approximately 0.5 µg/ml of RNA probe was placed on each section, and hybridization was performed at 50°C for 16 h. After hybridization, the slides were washed briefly in 5×sodium saline citrate (SSC; 1×SSC=0.15 M NaCl, 0.015 M sodium citrate) briefly and then washed in 50% formamide, 2×SSC at 50°C for 30 min. RNase A treatment (10 µg/ml) was carried out at 37°C for 30 min. The slides were then washed twice with 2×SSC and 0.2×SSC for 20 min at 50°C. Hybridized digoxigenin-labeled probes were detected with the aid of a nucleic acid detection kit (Boehringer Mannheim GmbH Biochemica, Mannheim, Germany) according to the manufacturer's instructions. Controls included (1) hybridization with the sense (mRNA) probe, (2) RNase A treatment (20 µg/ml) prior to hybridization, and (3) the use of neither antisense nor anti-digoxigenin antibody. All three controls showed no positive signals.

Reverse transcription polymerase chain reaction analysis

Total RNA was extracted from three of five cases of calcified tendon tissues (cases 1–3) using acid guanidine thiocyanate-phenol-chloroform (AGPC) methods [5] and the TRIzol reagent (Gibco Brl Life Technologies, N.Y.) according to the manufacturer's instructions. tRNA (1 µg) from each sample was reverse transcribed using Ready-To-Go You-Prime First-Strand Beads (Pharmacia Biotech Inc., N.J.) with an oligo-dT primer as directed by manufacturers. Thereafter, 1 µl of each reaction product was amplified using Ready-to-Go PCR beads (Pharmacia) with 12.5 pmol each of human OPN-specific primers (sense and antisense). Oligonucleotides used as human OPN-specific primers were as follows: human OPN 5'-GAAAGCGAGGAGTTGAATGG-3' (5' sense), and 5'-TTCCATGAAGCCACAAACTA-3' (3' antisense; 646–665 and 1168–1188, respectively, [37] Gene Bank database access no. J04765). The following PCR conditions were adopted in the present study. Thirty cycles were performed with Perkin-Elmer/cetus DNA Thermal Cycler (Takara Shuzo Co., Kyoto, Japan) at 94°C for 0.5 min, 58°C for 0.5 min, 72°C for 1 min, then 72°C for 7 min at the end of the procedure. The PCR products (10 µl) were electrophoresed in an agarose gel. Sequencing analysis was performed to compare the nucleotide sequence of the PCR product with human OPN.

Results

Immunohistochemistry

In all cases of caclicified tendinitis, specific staining for OPN was detected only in the cells adjacent to the calcified area. Morphologically, two distinct types of OPN-positive cells were observed (Fig. 2): mononuclear fibroblastic cells and round-shaped mutinuclear cells (Fig. 2A). The former were negative for CD68 and TRAP, whereas the latter were positive for CD68 and TRAP (Fig. 2B, C). No expression of OPN, CD68, and TRAP was demonstrated in cells located away from calcified lesions. No OPN was observed in cases of non-

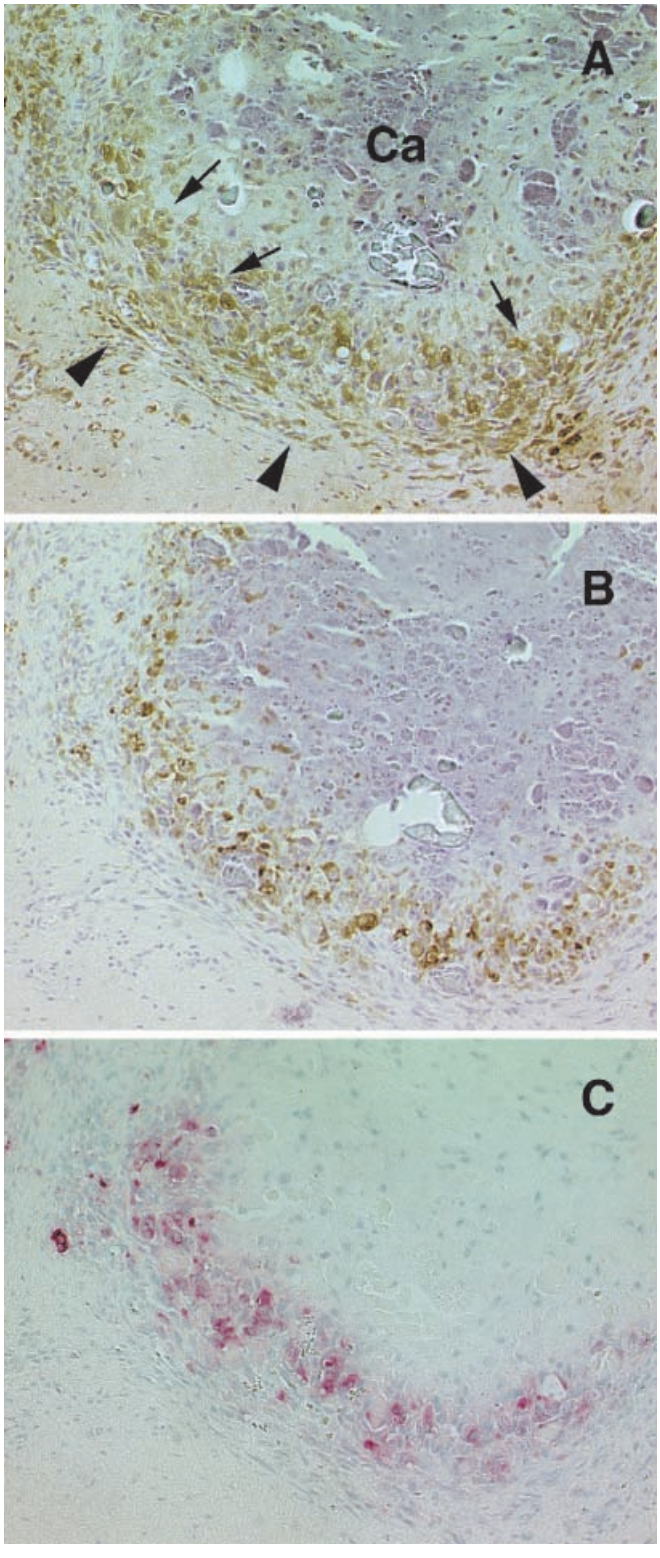

Fig. 2 Representative photomicrograms showing the calcified area in human calcified rotator cuffs. Paraffin-embedded sections were immunostained and counterstained with hematoxylin as described in Patients and methods. **A** Immunostaining of osteopontin (OPN) with a mouse monoclonal antibody against human OPN. Positive staining was observed in cells surrounding the calcified area. Round and multinucleated cells in the inner layer (*arrows*) and fibroblastic cells in the outer layer (*arrowheads*) were positive for OPN. **B** Immunostaining of CD68 with a monoclonal antibody against human CD68. Cells in the inner layer were positive for CD68. **C** Tartrate-resistant acid phosphatase (TRAP) staining. Cells in the inner layer were positive for TRAP. **A**, **B**, and **C** are serial sections. Original magnification ×400; *Ca* calcified area

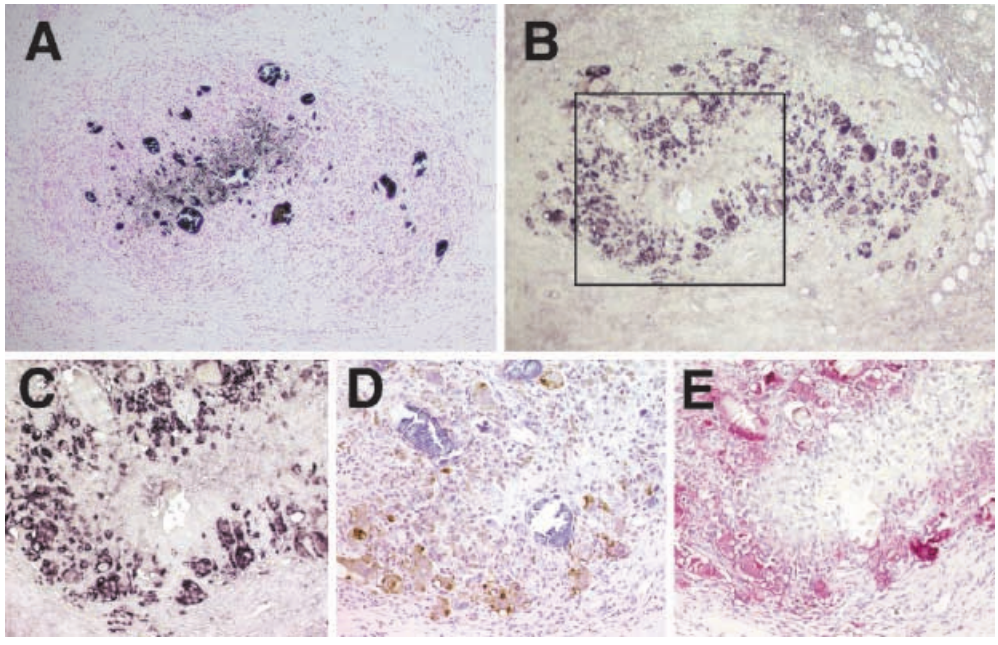

Fig. 3 Representative photomicrograms showing the calcified area in human calcified rotator cuffs. **A** von Kossa staining. *Black area* shows calcification. **B** In situ hybridization of osteopontin (OPN). OPN mRNA was detected in cells surrounding the calcified area. (**A** and **B** are serial sections. Original magnification ×100). **C** Higher magnification of the squared area in Fig. 2B. Multinucleated cells were positive for OPN mRNA. **D** Immunostaining of CD68 with a monoclonal antibody against human CD68. The section was couterstained with hematoxylin. Multinucleated OPN mRNA positive cells expressed CD68. **E** Tartrate-resistant acid phosphatase (TRAP) staining. Multinucleated OPN mRNA positive cells were TRAP positive. **C**, **D,** and **E** are serial sections. Original magnification ×200

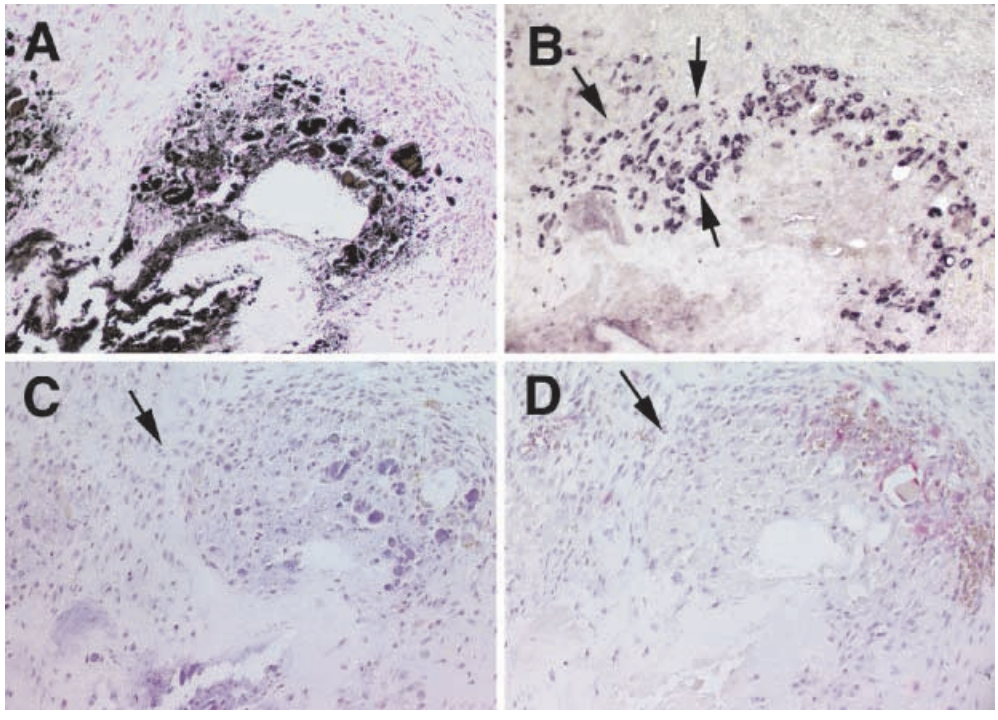

Fig. 4 Calcified area in human calcified rotator cuffs. **A** von Kossa staining. *Black area* shows calcification. **B** In situ hybridization of osteopontin (OPN). OPN mRNA was detected in fibroblastic cells (*arrows*) surrounding the calcified area. **C** Immunostaining of CD68 with a monoclonal antibody against human CD68. The section was couterstained with hematoxylin. Fibroblastic cells were negative for CD68 (*arrows*). **D** Tartrate-resistant acid phosphatase (TRAP) staining. Fibroblastic cells were negative for CD68 (*arrows*). **A**–**D** are serial sections; original magnification ×200

calcified tendon tissue taken from control patients (data not shown).

In situ hybridization

The distribution of cells expressing OPN mRNA was similar to that observed in the immunohistochemical study (Fig. 3 and Fig. 4). OPN mRNA was detected in cells adjacent to the calcified areas (Fig. 3A, B and Fig. 4A, B). Multinucleated cells positive for CD68 and TRAP (Fig. 3D, E) and fibroblastic cells negative for CD68 and TRAP (Fig. 4C, D) expressed OPN mRNA (Fig. 3B and Fig. 4B). Cells located away from calcified lesions did not express OPN mRNA and no OPN mRNA was observed in the non-calcified tendon tissue taken from the control patients (data not shown).

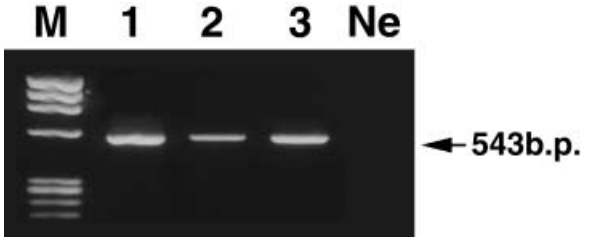

Fig. 5 Detection of osteopontin (OPN) mRNA using reverse transcriptase polymerase chain reaction (RT-PCR) analysis. RT-PCR products from three cases of calcified tendinitis (*lanes 1–3* cases 1–3) were electrophoresed in an agarose gel as described in Patients and methods. *Lane Ne* represents the negative control PCR reaction product without RT. *M* Marker

Reverse transcription polymerase chain reaction analysis

Human OPN mRNA was detected in all three samples of the calcified tendons as a band of predicted size (Fig. 5). Sequence analysis confirmed the nucleotide sequence of the PCR product to be that of human OPN.

Discussion

This is the first description showing the involvement of OPN in ectopic calcification in tendon tissues. The tendons were obtained at the surgery from patients with calcifying tendinitis, a major painful ectopic calcification occurring at the articular sites. The etiology of calcifying tendinitis of the rotator cuff tendons has been examined by several investigators but is still controversial. Codman proposed that degeneration of the tendon fibers precedes calcification, and Mohr and Bilger believed that the process of calcification starts with a necrosis of tenocytes with a concomitant intracellular accumulation of calcium [24]. In contrast, Uhthoff proposed that the calcification of tendon tissues is not due to a dystrophic calcification of the degenerative tendinous tissue but due to a cell-mediated reactive process [34]. However, the specific molecules relating to the pathomechanism of calcifying tendinitis had not been investigated prior to the present study.

OPN is believed to be a potent regulator of calcium deposition, and ours is the first report indicating the involvement of mineralization-related molecules in ectopic calcification in tendon tissues in humans. This study has demonstrated the presence of OPN in the tissues of calcifying tendinitis, which is the most common condition among ectopic calcification at articular sites. Both OPN mRNA and protein were localized in the cells surrounding the calcium deposits. OPN was not expressed in the cells located away from the calcified area. RT-PCR confirmed the actual synthesis of OPN at the molecular level in the calcified rotator cuff tissue.

The expression of OPN was limited to cells adjacent to the calcified area, and the present findings indicated that OPN appeared to be synthesized by two distinct types of cells, namely mononuclear fibroblast-like cells negative for CD68 and TRAP and TRAP- and CD68-positive macrophage lineage cells. The fibroblast-like cells may endogenously derive from tendon tissues and the macrophage lineage cells may arise exogenously under inflammatory conditions. Although the exact origin of these cells remains to be elucidated, the present data shows that at least these two types of cells are involved in the pathomechanism of calcifying tendinitis through the synthesis of OPN.

These findings are consistent with the previous investigations showing involvement of OPN in several pathological calcifications in humans, including vascular calcifications [8, 10, 12, 18, 27, 28, 32] and renal stones [2, 15, 20, 21, 33]. In these conditions, OPN is believed to not only facilitate cell migration and adhesion but to also regulate calcium deposition. As is the case with these pathological conditions, OPN may act as a regulator of calcium deposition in calcified tendinitis. OPN has been reported to be able to bind Ca^{++} at high capacity and low affinity in bone [4], but several in vitro studies suggest that in contrast, OPN inhibits calcium oxalate and hydroxyapatite growth [3, 6, 16, 17, 33]. Furthermore, OPN may contribute to resorption of calcium deposition, because OPN-positive multinucleated cells phenotypically resemble osteoclast. Such a possible role of OPN may possibly explain the clinical evidence of spontaneous resorption of calcifying tendinitis [35]. Thus, although the present study demonstrates the involvement of OPN in the pathomechanism of calcified tendinitis, a condition occurring in the soft tissues (not bone), the details of the mechanisms through which OPN regulate calcification in rotator cuffs remain to be investigated.

Finally, we have identified the possible cellular contribution to the pathomechanism of calcified tendinitis via OPN synthesis. The present findings will give us new insight into the molecular pathology of ectopic calcification, such as calcifying tendinitis, a common painful condition occurring in humans.

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