Involvement of multinucleated giant cells synthesizing cathepsin K in calcified tendinitis of the rotator cuff tendons


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Abstract

Objectives. Calcified tendinitis of the shoulder joint is a common painful condition. Resorption of the calcium deposits is one of the key events in the pathogenesis of this disease. The aim of this study was to examine whether the multinucleated giant cells that appear in this condition have osteoclast phenotypes.

Methods. Immunohistochemical and RNA in situ hybridization analysis of cathepsin K, a marker for osteoclasts, was performed in human surgical samples.

Results. The multinucleated cells located near the calcium deposits were positive for cathepsin K protein and mRNA. Reverse transcription–polymerase chain reaction using human cathepsin K-specific oligonucleotide primers confirmed that synthesis of cathepsin K mRNA occurs in the tissues of calcified rotator cuffs.

Conclusion. The multinucleated giant cells which appear in the resorption area of calcium deposits in calcified tendinitis have the osteoclast phenotype.

Key words: Rotator cuff tendon, Calcified tendinitis, Osteoclast, Cathepsin K.

Calcified tendinitis of the rotator cuff tendons is a painful heterotrophic calcification of the tendon tissue that commonly occurs in the shoulder joint [1, 2]. In the natural course of this disease, the final step is characterized by resorption of the calcium deposits, leading to relief of the persistent severe pain [3]. Thus, calcium deposit resorption is an extremely important event in this disease. However, the cellular mechanisms involved in this resorption are not well documented, particularly at the molecular level.

Previous histological analyses have demonstrated the existence of phagocytosing multinucleated cells in the resorption area [4]. However, these multinucleated giant cells have not been well characterized. In bone metabolism, i.e. bone remodelling and bone morphogenesis, an acidic environment is essential for the resorption of mineral deposition [5]. It is generally accepted that osteoclasts play an important role in establishing an acidic environment [5]. Thus, we hypothesized that the multinucleated giant cells which appear in calcified tendinitis resemble osteoclasts phenotypically.

Recent investigations have shown that cathepsin K, a lysosomal enzyme, is synthesized specifically by cells of osteoclastic lineage [6–9]. The present study examined the expression of cathepsin K in the cells that appear during the resorption of calcium deposits in calcified tendinitis of the rotator cuff tendons. Osteoclast-specific molecules, such as cathepsin K, were immunolocalized leading to relief of the persistent severe pain [3]. Thus, calcium deposit resorption is an extremely important event in this disease. However, the cellular mechanisms involved in this resorption are not well documented, particularly at the molecular level.

Materials and methods

Preparation of tissues

Rotator cuff tendon tissue containing a calcified region 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. Informed consent was obtained from all patients. Tissue samples were prepared as previously described [10]. They were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS) (pH 7.4) (Sigma, St Louis, MO, USA), and were dehydrated in an ethanol series and embedded in paraffin. Sections (5 μm) were made on a microtome, and the sections were stained with haematoxylin and eosin, and were also stained with von Kossa’s method.
to reveal calcium deposits. The residual serial sections were prepared for immunohistochemistry and in situ hybridization.

**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 [11]. A rabbit polyclonal antibody against the C-terminal polypeptide of mouse cathepsin K was purified by affinity chromatography, and reactivity with human cathepsin K was confirmed by Western blotting. The antibody was used as a primary antibody at a concentration of 1:100. Tissue sections were briefly deparaffinized and dehydrated and placed in 3% H$_2$O$_2$ 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 (rabbit) 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 (rabbit IgG; Nichirei, Tokyo, Japan) 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 then washed in PBS. Finally, a colour reaction was performed using the substrate 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Tokyo, Japan). Sections were counterstained with haematoxylin 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 substrate. These sections were also counterstained with haematoxylin.

**In situ hybridization**

In situ hybridization was carried out according to a method that we have described previously [10]. A human cathepsin K cDNA [residues 586–1180] was obtained by the polymerase chain reaction (PCR), and was subcloned into the pGEM-T plasmid. The plasmid was linearized with SacII and transcribed with SP6 RNA polymerase to generate an 0.595-kilobase antisense probe. The plasmid was also linearized with SpeI and transcribed with T3 RNA polymerase to generate a sense probe.

**RNA extraction and reverse transcription–PCR**

Total RNA was extracted from calcified tendon tissues from two patients by acid guanidine thiocyanate-phenol-chloroform methods using Trizol (Gibco, USA) according to the manufacturer’s instructions. One microgram of RNA from each sample was reverse-transcribed in 20 μl of a reaction mixture containing 400 U of Molony murine-leukaemia virus reverse transcriptase and 50 pmol antisense primer for human cathepsin K cDNA (GenBank accession number NM000396). Thereafter, 1 μl of each reaction product was amplified in 25 μl of a PCR mixture containing 0.125 U of Taq DNA polymerase and 12.5 pmol of each of the primers (sense and antisense). Oligonucleotides used for the reverse transcription (RT) and PCR were as follows: human cathepsin K, 5′-AAGAAGAAACTGGCAAACT-3′ (5′ sense), 5′-ATCGTTACCTGCACCATCG-3′ (3′ antisense) (nucleotides 586–605 and 1161–1180, respectively). The following PCR conditions were adopted in the present study. Thirty cycles were performed with a Perkin-Elmer/Cetus DNA Thermal Cycler (Takara Shuzo, 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. Ten microlitres of the PCR products were electrophoresed in an agarose gel.

**Results**

Histological analysis revealed that multinucleated TRAP-positive giant cells appeared adjacent to the calcium deposits (Fig. 2A and B). Cathepsin K was immunolocalized in cells of this type (Fig. 2C) and in situ hybridization revealed mRNA for cathepsin K in the multinucleated cells (Fig. 2D). RT-PCR products showed single bands at the expected molecular weights for human cathepsin K (Fig. 3). The identity of the cDNA obtained by RT-PCR to the nucleotide sequence of human cathepsin K was confirmed by sequence analysis.

**Discussion**

The present findings indicate that multinucleated giant cells express cathepsin K at both the protein and the transcriptional level. Moreover, combined with the positive staining for TRAP, these cells were confirmed to have the osteoclast phenotype. Expression of human cathepsin K mRNA was further supported by RT-PCR analysis. Little is known about the pathomechanisms involved in the resorption of mineral deposition in calcified tendinitis. The present data are the first to...
Fig. 2. Histological findings showing multinucleated giant cells in tendon tissues of calcified tendinitis. (A) Von Kossa staining: deposition of minerals (black areas) on the tendon tissues. (B) TRAP staining: multinucleated giant cells (arrows) show positive staining for TRAP. (C) Immunolocalization of cathepsin K. Multinucleated cells are immunostained by a rabbit polyclonal antibody against human cathepsin K (arrows). A–C are nearby sections. Magnification ×200. (D) RNA in situ hybridization. mRNA for human cathepsin K is localized in multinucleated cells (arrows). Nearby section to C. Magnification ×400.

characterize the multinucleated cells involved in the resorption of calcium deposits.

Cathepsin K is widely accepted to be a marker of cells of the osteoclast lineage [6–9]. Thus, the present data indicate that the multinucleated cells which appear near the site of calcium deposition in calcified tendinitis [4] phenotypically resemble osteoclasts. As observed in bone resorption [5], osteoclastic cells secrete proteins and contribute to the degradation of deposited minerals. Under this scheme, cathepsin K appears to be involved in the degradations of collagens, which are the major components of rotator cuff tendons.

Future studies should be directed towards elucidating the regulatory mechanisms involved in osteoclastogenesis and osteoclast function. Such advances may lead to new techniques to control and treat calcified tendinitis.

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