Externalisation of calpain (calcium-dependent neutral cysteine proteinase) in human arthritic cartilage


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Abstract

Objective
Calcium-dependent neutral cysteine proteinase (calpain) was originally referred to as an intracellular enzyme. However, recently it has come to be considered as an extracellular matrix proteinase, as well having a degrading effect on cartilage proteoglycan. In the present study we sought to determine whether human articular cartilage chondrocytes themselves have the capability to produce and secrete this interesting proteinase.

Methods
Human articular cartilage tissue cultures from osteoarthritic (11 specimens from 7 patients) and rheumatoid arthritis (3 specimens from 2 patients) knee joints were established, and the m-calpain released into the culture medium was concentrated and detected by immunoelectrophoretic blotting. The presence of m-calpain in the arthritic cartilage was also examined by immunohistochemistry before and after culturing.

Results
M-calpain was detectable in all of the cartilage tissue culture supernatants (conditioned medium) by western blotting. Positive intracellular immunostaining of m-calpain in chondrocytes was observed in all samples. Furthermore, m-calpain was found to be present in the matrix and on the articular surface of the cartilage in half of the specimens.

Conclusion
The findings of our experiment suggest that cartilage chondrocytes may actively take part in m-calpain production and that they may have the capacity to release it into the extracellular matrices.

Key words
Human, cartilage, calpain.
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Introduction

Articular cartilage destruction is the most significant pathologic process in osteoarthritis and rheumatoid arthritis. Although the precise biochemical mechanisms involved in cartilage breakdown are not completely understood, enzymes with the capacity to degrade cartilage matrix components, especially neutral metalloproteinases including collagenases (1, 2), stromelysins (3-5) and gelatinases (6, 7), are the most studied and suspected factors. However, other proteolytic pathways driven by cathepsins (8, 9), elastases (10) and a plasminogen activator (11) are also thought to contribute to this process.

Calpain is an interesting and unique cysteine proteinase, as it has a neutral pH optimum and is activated by calcium ions (12). There are two forms of calpain which have different calcium ion requirements for their activation: m-calpain is activated by micromolar and m-calpain by millimolar concentrations of calcium ions. Calpain, together with its specific endogenous inhibitor calpastatin, is known to be widely distributed in animal cells (13, 14) and to represent a distinct proteolytic system. Previously, calpain was classified as an intracellular enzyme (13, 15). However, current experimental evidence suggests that calpain can be characterized as a neutral matrix proteinase as well, and may contribute to cartilage destruction. Calpain has been detected in the synovial fluid and tissue of arthritic joints (16-18), in the extracellular cartilage matrix at the growth plate (19) and in fracture callus (20). Calpain secretion into the culture medium by synovial fibroblasts from arthritic patients has been demonstrated (18, 21) and the increased appearance of m-calpain in joint tissues including cartilage was also reported in experimental arthritis in mice (22, 23). The proteoglycan degrading capability of m-calpain has also been clearly demonstrated (24, 25).

Cartilage tissue culture provides an in vitro system which partially mimics the in vivo environment of cartilage and is a frequently used model for studying cartilage metabolism (7, 26-28). Using this method, the most intensively studied matrix metalloproteinases involved in cartilage destruction were shown to be secreted by cartilage chondrocytes (3, 7, 26-28). In the present experiment, we established short term tissue cultures of human articular cartilage from osteoarthritic (OA) and rheumatoid arthritic (RA) patients. The objective was to determine whether calpain is secreted by chondrocytes into the culture medium of cartilage explant, as well as to demonstrate the presence of calpain in human cartilage tissue.

Materials and methods

Cartilage tissue culture

Arthritic articular cartilage samples were obtained at the time of total knee replacements from 7 OA (11 specimens) and 2 RA (3 specimens) patients (ages 54-80 years). Full-thickness cartilage slices were kept wet and processed within 3 hours of their removal. No attempt was made to separate the areas of cartilage demonstrating different degrees of destruction. In each case, 1 g of wet weight cartilage was cut into small pieces, washed 3 times in serum-free Dulbecco’s Modified Eagle’s Medium (DMEM) containing 100 units of penicillin and 100 mg streptomycin per ml, and then incubated in 10 ml DMEM containing 10% fetal calf serum (FCS) and antibiotics at 37°C under an atmosphere of 5% CO₂ for 60 hours. The viability of the chondrocytes was evaluated by histology in hematoxylin and eosin stained sections. After the culture period, the conditioned medium was harvested and immediately subjected to further processing.

**DEAE-cellulose chromatography**

Conditioned medium was applied to 1.5 ml DEAE-cellulose columns (DE-52; Whatman, Springfield, England) that had been equilibrated with 20 mM Tris-HCl buffer, pH 7.5, containing 50 mM NaCl, 1 mM EGTA, 1 mM EDTA and 5 mM 2-mercaptoethanol. The column was washed several times with the same buffer, but also containing 200 mM NaCl. The protein was eluted with 2 ml buffer containing 400 mM NaCl. Chromatography was performed at 4°C. The eluent was finally concentrated 50-fold using a microconcentrator (Centricon-10; Amicon, Beverly, MA).
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**Immunoelectrophoretic blotting**
Polyacrylamide slab gel electrophoresis (PAGE) in the presence of 0.1% sodium dodecyl sulfate (SDS) was performed with 10% resolving gels and 3% stacking gels. After gel electrophoresis, sample proteins were transferred to a nitrocellulose membrane according to the methods of Towbin et al. (29). Nitrocellulose membranes were first incubated with rabbit anti-m-calpain antibodies that had been prepared and characterized to be specific for m-calpain as described (14, 19, 30), and then with a peroxidase-conjugated second antibody. Antigens were visualised by peroxidase staining according to the methods of Hawkes et al. (31) using diaminobenzate tetrahydrochloride (DAB) as substrate.

**Histology and immunohistochemistry**
Before and after the tissue culture period, 2 blocks of cartilage from each patient were obtained randomly for histology and immunohistochemistry. After 2 days of fixation in 10% buffered formalin at 4°C, 4 µm paraffin sections were prepared and stained with hematoxylin and eosin, and safranin O. For the immunohistochemistry, sections were deparaffinized and then several sections from each patient were pre-incubated with bovine testis hyaluronidase (3000 U/ml in phosphate buffer) for 1 hour. This was followed by blocking of the endogenous peroxidase by incubation with 3% H₂O₂ in H₂O for 5 minutes. Non-specific binding was blocked by incubation with 5% non-fat dry-milk in 50 mM Tris-HCl buffer, pH 7.8, containing 0.3 M NaCl and 0.1% Tween 20 (TBST) for 20 minutes followed by incubation with 3% bovine serum albumin (BSA) for 5 minutes. The slides were then incubated overnight with the anti-m-calpain antibody (the same used for immunoblotting) diluted at 1:800 in 5% non-fat dry-milk/TBST in the case of hyaluronidase predigested and at 1:500 in case of non-pretreated sections. Subsequently, the labelled streptavidin biotin (LSAB) (DAKO Japan Co, Ltd., Kyoto, Japan) method was used for immunostaining. Briefly, bound antibody was targeted for 10 minutes with biotinylated anti-rabbit immunoglobulin antibody (DAKO-E432), diluted 1:100 in TBST containing 1% BSA, and then incubated with peroxidase-conjugated streptavidin (DAKO-P397) diluted at 1:80 in TBST. The slides were developed by incubation with 0.05% DAB in the presence of 0.03% H₂O₂ in 50mM Tris-HCl, pH 7.6. The sections were finally counterstained with Mayer’s hematoxylin, dehydrated and mounted. All procedures were carried out at room temperature except for the overnight incubation with the first antibody, which was done at 4°C. Negative control specimens were processed simultaneously using normal rabbit serum (DAKO-x902) as the first antibody.

**Results**

**Demonstration of m-calpain in conditioned medium of arthritic cartilage by immunoelectrophoretic blotting**
After tissue culture of arthritic cartilage, m-calpain was concentrated in the conditioned medium. The samples were subjected to SDS-PAGE, and then immunoblotted with the rabbit anti-m-calpain antibody (Fig. 1). All the samples from arthritic cartilage demonstrated a clear positive band at 80 kd (lane OA 1-7b and RA 1-2b), which corresponds to the m-
Fig. 2. Representative photomicrographs of m-calpain immunostaining in arthritic cartilage. Positive intracellular immunoreactivity in the osteoarthritic (A) and rheumatoid arthritic (B) cartilage samples before, and after (C) the tissue culture period. Positive extracellular immunoreactivity is shown in the matrix and on the surface of the cartilage (D). No immunoreactivity was detected in control sections treated with non-immunised rabbit serum (E). (Hematoxylin counterstained, magnification x 400).
calpain large subunit. Samples prepared from the medium with 10% FCS without cartilage explants did not show any such band (lane M). Purified porcine kidney m-calpain was processed as a positive control and the large subunit demonstrated a positive band at 80 kd (lane C).

**Histological findings of the arthritic cartilage samples**

Arthritic cartilage samples from different patients demonstrated a wide range of histologic characteristics of cartilage destruction ranging from minimal changes to almost complete disorganisation. In some specimens the chondrocytes showed diffuse hypercellularity, whilst in others hypocellularity was observed. Safranin O staining demonstrated different degrees of proteoglycan depletion in the samples, ranging from moderate to severe reduction in proteoglycan staining in the cartilage matrix. Based on the histologic appearance of the chondrocytes before and after the tissue culture period, the cell viability was found to be preserved as there was no change detected in the hematoxylin and eosin stained sections.

**Immunohistochemical appearance and localisation of m-calpain in arthritic cartilage**

All of the osteoarthritic (Fig. 2A) and rheumatoid arthritis (Fig. 2B) cartilage samples demonstrated m-calpain immunostaining of chondrocytes both before and after (Fig. 2C) the tissue culture period. The frequency of cytoplasmic immunostaining of chondrocytes for m-calpain ranged between 10% and 90% in different cartilage samples and the immunopositive cells were found to be distributed in all layers of the cartilage. In half of the samples extracellular immunoreactivity of m-calpain was also observed in the matrix and on the surface of the cartilage (Fig. 2D). OA and RA specimens demonstrated similar characteristics of immunostaining. There was no difference in the immunostaining pattern before and after the tissue culture period. Extracellular m-calpain immunostaining was slightly fainter in the hyaluronidase-treated sections than in the non-treated series (data not shown).

Staining for m-calpain was absent in all of the sections incubated with normal rabbit serum as primary antibody (Fig. 2E).

**Discussion**

Breakdown of the extracellular matrix of articular cartilage is thought to be mediated by proteinases. Although the identity of these enzymes seems to be established, current experimental evidence suggests the possibility that calpain may also contribute to matrix degradation. M-calpain has been detected in joint tissues in experimental arthritis in mice (22, 23), and calpains have been demonstrated in the synovial tissues of arthritic patients (18, 21). Previously calpain was classified as an intracellular proteinase, but recently the extracellular detection of m-calpain was reported in the arthritic joint fluid (16-18, 23), the extracellular matrix of the growth plate (19), and in the fracture callus (20). Finally, calpain is a potent proteoglycanase, as it demonstrates a dose-dependent and limited proteolytic activity on proteoglycan (24), the major non-fibrillar extracellular component of cartilage.

An important question is: “Which cells are responsible for the production of the destructive enzymes in the synovial joints?” All of the synovial tissues have been demonstrated to be involved in the production and secretion of the major matrix metalloproteinases including the arthritic synovial lining and sublining cells, cartilage chondrocytes (3, 4, 32) and inflammatory cells (2, 6). In the case of calpain, it was clearly demonstrated that amongst the arthritic synovial lining cells, synovial fibroblasts (B cells) are responsible for calpain secretion (21). In experimental arthritis, it was observed that intra-articular inflammatory cells demonstrate cytoplasmic and surface calpain staining (23). Chondrocytes at the growth plate (19) and the fracture callus (20) were also shown to produce calpain.

In the current study we demonstrated the release of m-calpain into the conditioned medium of cartilage tissue cultures, its intracellular localisation in chondrocytes and its extracellular localisation in human cartilage matrix. The characteristics of calpain expression did not differ between our OA and RA cartilage samples, a fact which suggests a similar mechanism of calpain involvement in the cartilage metabolism of these two conditions. Although the contribution of the diffusion of previously synthesised m-calpain from the cartilage matrix into the culture medium must be considered, the significant cytoplasmic staining of m-calpain in cartilage chondrocytes suggests that the most likely source of the released m-calpain during the explant culture period was the cartilage chondrocytes. To further confirm these findings, studies using radio-labeling methods and killed chondrocytes will be necessary.

In summary, ours is the first study to provide experimental data and evidence supporting the hypothesis that m-calpain can be produced and released by human articular chondrocytes.

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