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CALPAIN: A NEW MATRIX PROTEINASE ?

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ABSTRACT

• *Calpain, originally considered to be an intracellular proteinase, was demonstrated to be present extracellularly in growth cartilage of Wistar rat and also in synoval fluids of osteoarthritic patients. Proteoglycan-degrading proteolytic activities of calpain were demonstrated in vitro and in osteoarthritic synovial fluid. These data plus some recent reports of other investigators suggest that calpain-calpastatin system may have an important role in the metabolism of the extracellular matrix, although the pathways of exterruilization of these intracellular proteinase remain unclarified.*

INTRODUCTION

• The enzyme capable of degrading macromolecules in extracellular matrix in the connective tissue is called matrix proteinase. The matrix proteinase is involved in matrix remodeling in which certain important biological phenomena take place. Such phenomena include: (I) Emigration of inflammatory cells out of blood vessels, through the basement membrane and underlying loose connective tissue, (II) Migration and proliferation of microvascular endothelial cells and fibroblasts during neovasculariza-tion, (III) Editing of excess matrix components during rapid connective tissue synthesis and assembly, (IV) Removal of obsolete matrix components from migration, signaling and assembly pathways, (V) Breakdown of connective tissue components coupled to synthesis during tissue expansion and growth, (VI) Destruction of extracellular matrix during inflammation, activation of connective tissue cells, and fibrosis. (1) The matrix proteinase is known to act as a lysosomal enzyme like cathepsin D on proteins taken up by endocytosis, or as a secretory proteinase like collagenase on extracellular matrix. (1)

Calpain, also known by the name of CANP (calcium-activated neutral proteinase), is a calcium-dependent neutral cysteine proteinase. There are two types of calpain depending on the difference in Ca^{2+} requirements during its activation: calpain I which is activated by low concentration of Ca^{2+} (laM) and calpain II which is activated by high concentration of Ca^{2+} (mM). The presence of calpastatin, a specific inhibitor protein, has been also uncovered. (2) So far, calpain has been known as an intracellular proteinase. (3,4) Our recent investigation, however, has suggested that calpain may also function as a matrix proteinase capable of degrading cartilage proteoglycan.

Calpain and calpastatin in calcifying zone of growth cartilage (5,6)

In growth cartilage, the cartilage is calcified and new bone is formed by the process of endochondral ossification. Endocondral ossification is an important mechanism observed in the process of formation of most of the bones of the body such as bones in the extremities and spine. Morphologically, the growth cartilage consist of several layers: resting zone, proliferating zone, hypertrophic zone, calcifying zone and the bone tissue of metaphysis in that order from cartilage to bone side. In the hypertrophic and calcifying zones, some proteinases have been found (7), suggesting their involvement in the calcification mechanism. It is presumed that proteoglycan accounting for most of the matrix of cartilage has the ability to bind to calcium and suppress calcification. In the calcifying zone, the proteoglycan, affected by action of proteinases, undergoes deformation, thus allowing calcification to progress (8).

Calpain, a proteinase which acts at neutral pH, is activated according to the concentration of calcium ion. If calpain is present in the calcifying zone of growth cartilage,

and has proteoglycan-degrading activity, progress in calcification will accompany an elevation of calcium concentration, followed by activation of calpain, degradation of proteogycan, discharge of calcium and activation of calpain in that sequence, thus forming a vicious cycle toward calcification. The current study was designed to elucidate this presumption.

Of knee joints collected from newborn and 3-day-old Wistar rats, growth cartilages of distal femur and proximal tibia were stained by an immunohistochemical technique. With the use of rabbit antiserum monospecific to the heavy subunit (80 kDa) of calpain I and II, the distribution of calpain was examined by the peroxidase method. In the epiphyseal cartilage of the rat, positive staining with anticalpain II antibody was observed in hypertrophic cartilage cells as well as in the surrounding cartialage matrix. In the resting zone, hypertrophic zone and metaphysis, however, positive staining was not observed. Positive staining with anticalpain I antibody was also not observed in any of the zones. With homogenates of metaphyseal cartilage obtained from the same 3-day-old Wistar rats, semicrude calpain was purified by DEAE-cellulose chromatography using caseinolytic activity as a parameter.

Peak fractions containing caseinolytic activity were run on an SDS-polyacrylamide gel, and identification was done by immunoblotting using the antiserum identical to that used in the immmunohistochemical staining. From these homogenates, calpain was able to be biochemically detected. Findings from such immunohistochemical and biochemical examinations suggested the presence of calpain in hypertrophic chondrocytes and surrounding cartilage matrix of growth cartilage. Calpain, with its cartilage proteoglycan-degrading activity, which will be described in the following section, could be considered as one of the proteinases taking part in calcification of growth cartilage.

Degradation of cartilage proteoglycan by calpain (9)

The ability of calpain to degrade proteoglycan was examined with the use of calpain II extracted from growth cartilage or the kidney of rats and swine, and proteoglycan from articular cartilage of swine (proteoglycan monomer, proteoglycan aggregate and link protein (10) as substrates. These substances were further examined for changes in molecular size with the combined use of Sepharose 2B gel filtration chromatography, sucrose density gradient ultracentrifugation and agarose polyacrylamide gel electrophoresis. Findings were summarized as follows: (I) Decrease of molecular size was observed in proteoglycan monomers, proteoglycan aggregates and link proteins at neutral pH and calcium dependently with trace amount of the enzyme. (II) An inhibitory study confirmed that the above-mentioned effects were produced by calpain. (Ill) Besides such decrease of molecular size of proteoglycan monomers, observed were effects on molecular functions of proteoglycan, including disappearance of the abillity to reaggregate with hyaluronic acid.

Calpain and calpastatin of synovial fluids of osteoarthritis (11)

Once calpain was found to have a strong effect on degradation of cartilage proteoglycan, our interest shifted to whether calpain and calpastatin are present in synovial fluids or not. Because if calpain activity can be observed in synovial fluids, (I) the matrix of articular cartilage, which consists mostly of proteoglycan, would come in contact with calpain, thus possibly causing calpain to take part in degradation of the matrix of articular cartilage, and (II) an extracellular presence of calpain would be proved.

Synovial fluid of ossteoarthritis was selected for the material to prove the extracellular presence of calpain in synovial fluid, because the use of synovial fluid obtained from subjects with rheumatoid arthritis which is rich in cellularity would not be proper for this purpose. Cellularity of the synovial fluid of osteoarthritis is known to be poor. We nevertheless examined the activity of the synovial fluid which had been further processed by filtration so as to prevent contamination by calpain and calpastatin from cellular component. By passing a linear gradient of sodium chloride through the synovial fluids absorbed to DEAE-cellulose, peaks of caseinolytic activity were observed at the positions of 150 and 300 mM. The inhibitor study and immunoblotting using monospecific antiserum proved these peak fractions to be calpain I and II, respectively. With the use of peak fractions of calpain I and II obtained from the synovial fluids by DEAE-cellulose chromatography, cartilage proteoglycan-degrading activity was assayed: this activity was proved to be subjected to a nearly 100 % inhibition by E64 (a cystein proteinase inhibitor) and calpastatin (a specific inhibitor of calpain). Calpastatin was observed as a heat-stable calpain inhibitor at the position of 120 mM sodium chloride concentration on DEAE-cellulose chromatography of synovial fluids, and identified by immunoblotting using anticalpain antibody. Of synovial fluid samples used in the curent study, calpain I was superior to calpain II in 6 samples in which quantitative assay could be carried out, and the quantity of inhibitor activity was superior to the total quantities of activity of 2 calpains therefore, the total activity of synovial fluids, the inhibitor was quantitatively superior. Calpain and calpastatin were found to be present in synovial fluids of osteoarthritis while remaining active extracellularly.

A study recently published by other investigators also found calpain and calpastatin to be present in synovial fluids of rheumatoid arthritis. (12) Although there is a problem that if the balance of the calpain-calpastatin system is inclined to degradation or inhibition at the cartilage in osteoarthritis and rheumatoid arthritis, the matrix of articular cartilage consisting mostly of

proteoglycan would be in contact with the proteoglycan degrading activity-possessing calpain. Therefore, it is highly possible that calpain is involved in degeneration of articular cartilage in such diseases. Further investigations would be required on changes in calpain and calpastatin of synovial fluids in various articular diseases.

So far, two types of matrix proteinases have been known: proteinases which are secreted outside the cell throgh the Golgi body, such as collagenase, and lysosomal enzymes (a typical example is cathepsin D). These proteinases secreted from the inside to the outside of the cell are known, in general, to have signal peptide which determines transportation pathway between intracellular compartmens. Calpain is not a. lysosomal enzyme, and signal peptide is not contained either in calpain or in calpastatin.(13,14,15,16,17,18) Also known is the presence of proteins such as interleukin 1, which has no signal peptide, yet exerts its effects after moving outside the cell. (19) The pathway which interleukin 1 takes to come out of the cell without the help of signal peptide remains unsolved. Yet it is believed that during its move from the cell, the decrease of molecular size of interleukin 1 takes place simultaneously^ 19) It is unknown whether calpain and interleukin 1 share a common mechanism of secretion or not.

Calpains have been generally known as intracellular proteinases and catalyse selective but limited proteolytic modification of proteins. Findings from the current study suggested that calpain is present with an extracellular activity, and it has an extracellular role of proteoglycan-degrading activity. Some previous studies have demonstrated an extracellular localization of calpain in the skeletal muscle after sciatic denervation or starvation of rats by immunogold electronmicroscopy.(20) Although the pathway which calpain takes to move out of the cell is not known yet, it would be encouraging to carry out further studies on calpain because of its provision of such properties of matrix proteinase that it is present outside the cell in possession of enzyme activity and can degrade matrix proteoglycan.

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