IMMUNOCYTOCHEMICAL DEMONSTRATION OF CALPAIN IN SYNOVIAL CELLS IN HUMAN ARTHRITIC SYNOVIAL JOINTS

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ABSTRACT
Calpain is a calcium-dependent neutral cysteine proteinase. As a matrix proteinase, calpain has a degrading effect on cartilage proteoglycan. We have demonstrated that the protein is highly expressed in human arthritic synovial joints and therefore may play an important role in cartilage degradation. In the present study, an attempt was made to examine whether particular phenotype of synoviocyte is associated with the expression of calpain. Non-inflamed, rheumatoid and osteoarthritic synovia and skin were analysed as tissue sections and in cell culture, by double immunostaining with an anticalpain antibody and a monoclonal antibody directed against macrophage antigens (DAKO CD68, PG-M1), and also by immunoblotting techniques. Marked differences were found in synovial lining cells by using double labeling techniques. Rheumatoid synovium showed the majority of calpain-positive cells; PG-M1-positive cells were clearly separated and there was little overlap. Osteoarthritic synovium showed almost the same results. In culture studies, immunoblotting using the anticalpain antibody demonstrated that calpain was present in conditioned media (supernatant) obtained from cultured dermal fibroblasts, as well as in the dermal fibroblasts themselves. These findings suggest that cells responsible for the synthesis of calpain largely represent the phenotype of synovial fibroblasts (B cells), but not of phagocytic synovial macrophages (A cells).

The rheumatoid synovium is heterogeneous. Although there is a debate about the distinction of synovial lining cells, originally named A and B cells by Barland et al. (3), accumulated evidence indicates that synovial lining cells contain two cell populations with different origins and functions (24). Phagocytic synovial macrophages (A cells) appear as mature monocyte-derived macrophages (4, 9, 10, 17, 41). Synthetic synovial fibroblasts (B cells) resemble fibroblasts (3), but their relationship to other fibroblasts is not well known.

Calpains (calcium-activated neutral protease, EC 3.4.22.17) are a widely distributed family of cysteine protease (18). μ-Calpain, or calpain I, is activated by μM Ca$^{2+}$, while m-calpain, or calpain II, requires mM Ca$^{2+}$ for activation (26, 27, 37). Along with their endogenous inhibitor calpastatin (29), both enzymes are found in most tissues including synovial tissues (42). Each calpain consists of a catalytic subunit (80 kd) which is distinct for each calpain, and a smaller (30 kd) subunit which is common to both calpains (26). Recently, the presence of an extracellular calpain-calpastatin system has been demonstrated in the calcifying zone of growth cartilage (33), in fracture callus of rats (28), and in osteoarthritis (OA) and rheumatoid arthritis (RA) synovial fluid in humans (12, 35). Our previous studies have shown that calpain degrades cartilage proteoglycan in vitro (33, 36). It is possible that calpain functions as a matrix proteoglycanase, facilitating articular cartilage degradation in RA and OA, since its action is highly regulated by calcium ion concentration and it has enzymatic activity at
neutral pH.

In previous studies, we have reported that RA synovium demonstrated specific cytoplasmic calpain staining in cells in the synovial lining layer (42). Cells responsible for synthesis of stromelysin have the phenotype of synovioblast (B cells) which is demonstrated by immunoelectron microscopy (30). Synthesis of synovial fluid hyaluronan has been ascribed to the type B lining cell (6, 8). However, TNFα has been produced by A cells (7). Wilkinson et al. (40) have recently reported that it is possible to identify by light microscopy a fibroblast subtype in the intima.

The objective of the present investigation is to establish what types of synovial lining cells are positive for calpain.

**MATERIALS AND METHODS**

**Tissue Specimens**

Inflamed synovium was obtained from patients with osteoarthritis or RA, definite or classic, according to the criteria of the American College of Rheumatology, formerly, the American Rheumatism Association (2), who were undergoing therapeutic joint surgery (knee, hip or elbow). Non-inflamed synovium was obtained from patients with joint trauma. Tissues were taken from clinically normal patients and showed no histological evidence for disease. Specimens were routinely fixed in 10% neutral formaldehyde, embedded in paraffin and sectioned onto poly-L-lysine or silane coated microscope slides at a thickness of 6 μm. Sections from two tissue blocks were studied in each case.

**Cell Culture**

Synovial tissue, obtained from patients with RA, OA, posttraumatic arthritis undergoing either therapeutic synovectomy or joint replacement, was finely minced and dissociated by treatment with bacterial collagenase and testicular hyaluronidase (Sigma, St. Louis, MO) according to the method of Hamerman et al. (13). Human skin fibroblasts were obtained from surgery specimens using an explant culture method. Synovial and skin fibroblasts were maintained in vitro in a 100 mm culture dish in Dulbecco’s modification of Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and the antibiotics, streptomycin (100 μg/ml) and penicillin (100 units/ml). U937 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD). These cells were maintained in a continuous culture in RPMI 1640 (GIBCO Laboratories, Grand Island, NY) culture medium, supplemented with 10% FBS, penicillin (100 units/ml), streptomycin (50 μg/ml). These cells were differentiated as previously described (21), by incubating the cells for 72 h with various agents, including retinoic acid (1.0 × 10⁻⁶ M), vitamin D₃ (1.0 × 10⁻⁸ M), and the phorbol ester TPA (1.6 × 10⁻⁷ M). This cell line was originally established from the pleural effusion of a patient with generalized, diffuse histiocytic lymphoma (34). Peripheral blood mononuclear cells were isolated from fresh, circulated venous blood collected from normal, consenting volunteers by Percoll density centrifugation as described previously (14). Monolayers of monocytes adherent to the 100 mm culture dish were then cultured in RPMI 1640 medium containing 10% FBS, penicillin, streptomycin. The U937 cells were cultured in the same medium. All cell types were maintained at saturated humidity in an atmosphere of 95% air/5% CO₂.

For the experiments, synovial and dermal fibroblasts were plated in 100 mm tissue culture dishes at a density of 4 × 10⁵ cells in 10 ml of DMEM containing 10% FBS and antibiotics and U937 cells (1.4 × 10⁵ cells) and peripheral blood mononuclear cells (3 × 10⁶ cells) in 10 ml of RPMI 1640 containing 10% FBS and antibiotics. The supernatant (after medium change) was centrifuged at 1,500 rpm for 5 min, and 0.5 ml of the supernatant was used for the lactate dehydrogenase (LDH) measurement and the res: (9.5 ml) was used for immunoblotting. For immunoblotting, synovial, dermal fibroblasts, U937 cells and peripheral blood mononuclear cell layers in culture were collected, suspended in sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis (SDS-PAGE) sample buffer, and sonicated on ice.

**Cell Viability**

Cell viability was determined by trypan blue dye exclusion and by the measurement of LDH (42).

**DEAE-Cellulose Chromatography**

Conditioned medium (supernatant) from cultured synovial cells, dermal fibroblasts, U937 cells and peripheral mononuclear cells were applied to a DEAE-cellulose column (1.0 × 2.5 cm) that had been equilibrated with buffer A (20 mM Tris HCl, pH 7.5, with 50 mM NaCl, 1 mM EDTA, 1 mM
EGTA, and 5 mM 2-mercaptoethanol). The column was washed thoroughly with the same buffer containing 20 mM NaCl and was eluted step-wise with 2 ml of the same buffer containing 400 mM NaCl, and then concentrated in Centricon 10 devices (about 40-fold concentration).

**Monoclonal Antibodies for Macrophages**

The monoclonal antibodies (MAB) used in the present study for the type A cells in the synovial membrane comprised HAM-56 (1), KP-1 (CD68) (31), EBM11 (CD68) (19), and PG-M1 (CD68) (11) (Dako Japan). The immunohistological staining patterns of MAB in different tissues are summarized in Table 1. The results were all obtained using a range of dilutions of each antibody to exclude differences due to sub-saturating concentrations of antibody.

**Immunohistochemical Analysis**

Calpains I and II were purified from human red blood cells and porcine kidney, respectively, as previously described (15, 20). The antibodies against calpains I and II have been shown not to cross-react (43). Paraflin sections were deparaffinized at room temperature followed by hemode (Fisher) and ethanol. Sections were rehydrated in water and transferred to 0.05 M Tris-buffered saline (TBS), containing 0.3 M NaCl and 0.1% Tween, pH 7.8. Due to low background staining, paraflin sections required blocking of endogenous peroxidase by incubation for 5 min at room temperature with 3% H₂O₂ and blocking of nonspecific second-antibody binding by incubation for 5 min at room temperature with normal bovine serum albumin. The slides were then incubated with the anti-calpain II antibody (1:800) overnight at 4°C. Immnoperoxidase staining was performed using the labeled streptavidin biotin (LSAB) method (Dako-k680 kit, Dako Japan) (23). Briefly, staining of the anti-calpain II antibody was carried out using the second step link antibody (biotinylated anti-rabbit and anti-mouse immunoglobulins) and horseradish peroxidase-conjugated streptavidin and DAB (3,3’-diaminobenzidine tetrahydrochloride) as substrate. Control slides were treated using normal rabbit serum.

**Double Labeling**

Double immunohistochemical labeling was performed by repeating the LSAB staining technique. After staining of the anti-calpain II antibody was carried out, the slides were treated with 0.1% trypsin (Sigma, 50 mM Tris-HCl, 0.1% CaCl₂, 37°C, 20 min) to disaggregate methylene complex due to formalin fixation. After trypsin treatment, the slides were blocked with normal bovine serum albumin and then incubated with MAB directed against human macrophage-associated antigens (CD68, PG-M1; 1:100; Dako) for 20 min at room temperature, followed by 10 min incubations first with the link antibody (biotinylated anti-rabbit and anti-mouse immunoglobulins) and then with alkaline phosphatase-labeled streptavidin (D396, Dako). The slides were then developed with New Fuchsin (K698, Dako) as substrate. Mayer’s hematoxylin or methyl green was used as a counterstain.

<table>
<thead>
<tr>
<th>Cell types</th>
<th>Antibodies</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>HAM-56</td>
</tr>
<tr>
<td>Macrophage, histiocyte</td>
<td>+</td>
</tr>
<tr>
<td>Kupffer’s cell</td>
<td>+</td>
</tr>
<tr>
<td>Intercamititating cell</td>
<td>+</td>
</tr>
<tr>
<td>Langerhans’ cell</td>
<td>–</td>
</tr>
<tr>
<td>Monocyte</td>
<td>+</td>
</tr>
<tr>
<td>Plasma cytomide cell</td>
<td>+</td>
</tr>
<tr>
<td>Myeloid precursor cell</td>
<td>–</td>
</tr>
<tr>
<td>Granulocyte</td>
<td>–</td>
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<td>References</td>
<td>1</td>
</tr>
</tbody>
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Staining: positive (+), weak (±), and negative (−)
Rheumatoid synovial cells and human peripheral monocyte-derived macrophages grown on Lab-Tek slides were fixed in 3.5% paraformaldehyde for 30 min and rinsed with TBS and subsequently incubated with anticalpain II antibody and PG-M1 in the same manner as for the synovial tissues.

**Protein Concentration**

Protein concentrations were determined by the microassay procedure (Coomassie Protein Assay Reagent; Pierce) (22), using bovine serum albumin as the standard.

**Immunoblotting**

PAGE in the presence of 0.1% 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 method of Towbin et al. (38). Nitrocellulose membranes were cut into strips and some of them were stained with 0.1% amide black in 40% methanol/10% acetic acid. The other strips were first incubated with anticalpain II antibody and then with a peroxidase-conjugated second antibody directed against the first antibody. Antigens were visualized by peroxidase staining according to the method of Hawkes et al. (16) using DAB as substrate.

**RESULTS**

**Monoclonal Antibodies for the Type A Cells in the Synovial Membrane**

In preliminary experiments, we investigated which antibody should be adopted to mark type A cells in the synovial membrane using the LSAB method.
Table 2. The Intensity of Staining for Calpain II and PG-M1 in Synovial Lining Layer from Patients with Rheumatoid Arthritis, Osteoarthritis and Non-Inflammatory Joint Disease

<table>
<thead>
<tr>
<th>Patients</th>
<th>Number</th>
<th>Calpain II</th>
<th></th>
<th>PG-M1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+++</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>Rheumatoid arthritis</td>
<td>27</td>
<td>17</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>Osteoarthritis</td>
<td>15</td>
<td>4</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>Non-inflammatory joint disease</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Staining: ++++, intense; ++, moderate; +, weak; --, none. Individual tissue samples were graded according to the intensity of staining.

PG-M1 (Fig. 1A) and KP-1 (Fig. 1B) showed an intensified staining pattern in RA synovial membrane, whereas HAM-56 showed weak staining (Fig. 1C). Control slides obtained by omitting the incubation with the first antibody did not show any staining (Fig. 1D). However, KP-1 recognizes not only macrophages but also some antigen-presenting cells and myeloid precursor cells (31). EBM11 can not detect monocyte-macrophage lineage cells on the formalin-fixed paraffin sections (19). Therefore, we used PG-M1 for the type A cells in the synovial membrane in further experiments.

Findings of Double-Labeling Studies

The intensity of staining for calpain II and PG-M1 in the synovial lining layer is summarized in Table 2. In non-inflamed tissues, the majority of PG-M1-positive cells was present in the normal layer of synoviocytes immediately adjacent to the joint space. To a lesser degree, cells with the PG-M1-positive phenotype were also present in the fibrous tissue and in the perivascular regions. However, no cells in these healthy synovial membranes showed staining with the antibody against calpain II (Fig. 2A, Table 2). In diseased tissue, the synovial lining layer contained cells bearing PG-M1 antigens (red staining) as well as those of calpain II (brown staining) in rheumatoid samples. Usually, these cells had a strong staining intensity to each antigen. Of special interest was the observation that cells positive to PG-M1 antigens were located at the immediate surface of the synovial membrane, but calpain-positive cells were present in the deeper areas of the synovial lining layer (Fig. 2B). Controls using non-immunized rabbit serum and PG-M1 showed no calpain II staining. PG-M1-positive cells were present in the layer of synoviocytes and in the immediate neighborhood of the superficial synovial membrane. Some positive cells were scattered in the subsynovial tissue (Fig. 2C). In osteoarthritic samples, calpain staining varied, with some areas of the sample similar to that of non-inflamed tissue and other areas similar to that of RA. However, PG-M1-positive cells were found at the most superficial lining layer (Fig. 2D). The intensity of immunostaining was slightly weaker in OA than in RA samples (Table 2). In general, in diseased synovial tissues, double labeling for calpain and PG-M1 showed a clear segregation of cells. Calpain II staining was seen in the basal part of the lining, whereas the most superficial lining cells showed PG-M1-positive staining, which is characteristic of mature macrophages.

In cultured, nonstimulated rheumatoid synovial cells (first passage), two types of cells were seen. One type showed calpain-positive cells (brown staining), the other type showed PG-M1-positive cells (red staining) (Fig. 3A). Calpain II was present in areas of the cytoplasm. In many calpain-positive cells, the cytoplasmic region showed intense staining with the anticalpain antibody. Similarly, PG-M1 antigens were present in the cytoplasmic region, but the PG-M1-positive cells were largely round-shaped with surface protrusions and had noncentrally located nuclei, and contained a large number of vacuoles which were characteristic of macrophages. Some areas of the cytoplasm with PG-M1-positive staining were masking for calpain II, but other areas are clearly stained with only PG-M1. In many synovial cells, calpain-positive cells and PG-M1-positive cells (which contain small amounts of calpain II) were clearly separated (Fig. 3A). Calpain staining was less intense with normal
rabbit serum and PG-M1, which served as a control for immunostaining (Fig. 3B). On the other hand, in peripheral monocyte cultures, many round-shaped cells showed PG-M1-positive staining (red) of the cytoplasm. However, some areas of these cells were also stained with calpain II (brown). Monocyte-derived macrophages had a similar pattern of staining to macrophages from the synovium (Fig. 3C).

**Immunoblotting of Calpain**

Synovial fibroblast samples, synovial fibroblast culture supernatants, dermal fibroblast samples and dermal fibroblast culture supernatants eluted and concentrated as described in the Materials and Methods sections were subjected to SDS-PAGE. The 80 kd heavy subunit of calpain II was found in synovial fibroblast samples and synovial fibroblast culture supernatants. Both the dermal fibroblast samples and cell culture supernatants from RA and normal patients showed 80 kd calpain bands on immunoblotting with anti-calpain II antibody, whereas the U937 cell culture (incubated with either retinoic acid, vitamin D3 or TPA) and peripheral blood mononuclear cell culture (mono-
cyte-derived macrophage) supernatants showed no calpain II band (Fig. 4).

DISCUSSION

Calpain was originally thought to be an intracellular proteinase (27). However, recent studies have demonstrated the extracellular calpain-calpastatin system in the calcifying zone of growth cartilage (33) and in fracture callus of rats (28), as well as in OA and RA synovial fluid in humans (12, 35).

Our previous study demonstrated that RA and OA synovial lining cells have specific cytoplasmic staining of calpains. In the present study, we attempted to know which type of synovial lining cells express calpain in human arthritic synovial joints.

The cells making up the lining of the synovium have long been known as type A and B synoviocytes. However, an intermediate form (type C syno-
viocytes) is sometimes seen, which is considered to have features of both A and B cells. Accumulating evidence shows that type A cells are macrophages and type B cells are fibroblasts (32). A cells account for approximately 30–50% of eluted cells from patients with RA and also from patients with non-inflammatory joint disease. B cells form about 10–13% of synovial lining cells in patients with RA, but are only infrequently encountered in patients with non-inflammatory joint diseases. The rest are exclusively classified as B cells (5).

Several monoclonal antibodies have been generated that preferentially label monocyte-macrophage cells. These include some monoclonal antibodies, such as HAM-56, KP-I (CD68), EBM11 (CD68), and PG-M1 (CD68). We used three antibodies, which detect the same single chain protein (CD68). Unlike many other CD leucocyte antigens, the CD68 molecule is antigenically very heterogeneous. This antigenic heterogeneity within the CD68 cluster could reflect either variations in the carbohydrate side chain of the antigen or differences in the folding of the central peptide core in different cell types (11). However, one of the CD68 antibodies, termed EBM11, can not detect monocyte-macrophage lineage cells in the formalin-fixed paraffin sections (19). Since KP-I recognizes not only macrophages but also some antigen-presenting cells and myeloid precursor cells (Table 1) (31), a preliminary experiment was conducted to investigate which antibody should be adopted as a marker for the type A cells during our experiment. Results showed that PG-M1, which reacts with a glycoprotein with a molecular weight of approximately 110 kd (11), showed a very specific and intensified staining pattern in synovial tissues (Fig. 1). The antigen is expressed primarily as an intracytoplasmic molecule, probably associated with
lysosomal granules. For this reason, we used PG-M1 as a marker for the type A cells in the synovial lining cells.

In the present study using double staining with anticalpain antibody and CD68, PG-M1 MAb, we demonstrated that in diseased synovium the majority of calpain-positive cells and PG-M1-positive cells were clearly segregated and there was little overlap (Fig. 2, B and D). Calpain-positive cells were located in the deeper part of the synovial lining cell layer immediately bordering the underlying fibrofatty connective tissue, while PG-M1-positive cells were in the superficial layer of synoviocytes. These findings correspond to the evidence that the type A cells are closely arranged above the type B cells in the synovial lining layer (32). Similarly, in cultured, nonstimulated primary synovial cells (first passage) preparations, calpain-positive cells and PG-M1-positive cells (although they may contain a small amount of intracellular calpain) were clearly separated (Fig. 3A). This finding shows that cultured primary synovial cells consist of type A cells (PG-M1-positive cells) and type B cells (calpain-positive cells). However, cultured synovial cells after two passages were largely positive for calpain, but lacked PG-M1-positive staining. This supports the fact that passing rheumatoid synovial cells are devoid of macrophages (25, 39).

Immunoelectron microscopy showed that cells responsible for synthesis of stromelysin have the phenotype of synoviocytes (B cells), but not of phagocytic synovial macrophages (A cells) (30). The type A cell is more numerous and contains a prominent Golgi apparatus, numerous vacuoles containing amounts of a dense granular material, many filopodia, mitochondria, intracellular fibrils, and micropinocytotic-like vesicles. The type B cell contains large amounts of endoplasmic reticulum
with fewer large vacuoles, micropinocytotic-like vesicles, and mitochondria (3). Different methods have been used for studying localization of antigens. However, immunohistochemical techniques of light microscopy using double staining is easier than that of immunoelectron microscopy in that we can use formalin-fixed paraffin sections. Therefore, we can easily distinguish different cells using color staining with this simple method.

Conditioned medium from cultured dermal fibroblasts, as well as the dermal fibroblasts themselves, obtained from RA and normal tissues showed a large subunit of calpain II (80 kd) by immunoblotting in culture (Fig. 4, lanes 4–7). The extra bands (below 80 kd) seen with immunoblotting in Fig. 4 (lanes 5 and 7) are probably due to degradation products of calpain II. These data clearly showed the presence of 80 kd calpain II. These findings are in line with the presence of calpain in synovial cell cultures (after second passages) which are devoid of macrophages (42). Since one of the primary characteristics of inflamed synovium is the presence of large numbers of activated macrophages, it was of interest to know if these cells can produce calpain in vitro. To answer this question, we performed studies using U937 cells and human
peripheral blood mononuclear cells as a control, because the type A synovial cell is a bone marrow-derived cell of the monocyte/macrophage series which has migrated to the synovial surface from local blood vessels. These cells do not proliferate in the synovial membrane (32). Log-phase cells were seeded into culture dishes and terminally differentiated into monocyte/macrophage-like cells (21), by incubating with either retinoic acid, vitamin D₃ or TPA. Peripheral blood mononuclear cells differentiated into macrophages. Under none of the experimental conditions tested were we able to detect calpain produced by macrophages (Fig. 4, lanes 8 and 9). There is no direct evidence that PG-M1-positive type A cells (which probably contain a small amount of intracellular calpain) do not produce calpain to the extracellular environment. However, in double labeling studies we showed that human peripheral blood monocyte-derived macrophages had patterns of staining for calpain II and PG-M1 similar to type A cells (Fig. 3, A and C). These findings strongly suggest that type A cells which would have properties similar to those of monocyte-derived macrophages do not produce this protease. The type B cell resembles a fibroblast, but its relationship to other fibroblasts is not clear. However, these results strongly support the idea that the type B cells of the synovia are the major source of calpains, because there are a small number of type C cells which have features of both A and B cells in the synovial lining layer.

In conclusion, it is conceivable from our findings that type B cells of the synovial lining layer are largely responsible for the production of calpain, which has a degrading effect on matrix proteoglycans in human arthritic synovial joints.

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