Utilization of a Recombinant Substrate rAgg1 to Study the Biochemical Properties of Aggrecanase in Cell Culture Systems*

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This paper describes the first report of the production and use of an artificial recombinant protein substrate to study “aggrecanase” activity. The substrate (rAgg1) is composed of the complete interglobular domain (IGD) of human aggrecan flanked by the “marker” sequences FLAG™ at the amino terminus and the human immunoglobulin G1 constant region at the carboxyl terminus. The expressed protein occurs as large multimolecular aggregates (>120 kDa) that, upon reduction, consist of a major isoform of 72 kDa (containing the IGD) and a minor 39-kDa species that through alternative splicing has had the IGD deleted. Using this recombinant substrate we developed a novel agarose cell culture system containing either rat chondrosarcoma or bovine chondrocytes that could be used in studies of the biochemical characterization of aggrecanase activities. These studies showed the following. (i) rAgg1 is a suitable substrate for aggrecanase proteolysis. (ii) Aggrecanase activity was specifically induced by exposing chondrocytes to retinoic acid. (iii) A considerable time period was required to synthesize and/or activate aggrecanase, with considerable differences in that found in rat chondrosarcoma versus bovine chondrocyte cultures. (iv) Aggrecanase cleavage of the aggrecan IGD does not require the presence of the G1 or G2 globular domains or keratan sulfate post-translational modification in the IGD. (v) Aggrecanase is a diffusible activity that does not require association with the chondrocyte plasma membrane or immediate pericellular matrix for its action.

Mechanisms of proteoglycan breakdown in connective tissue are complex and involve multiple agents and pathways. In studies investigating the catabolism of aggrecan, the experimental systems used have included monolayer cultures of primary chondrocytes (1), established chondrocyte cell lines (2, 3), and explant cultures using cartilage from a variety of anatomical sites and animal species (4–6). Cytokines such as interleukin-1 and tumor necrosis factor have been extensively used as agents that promote the degradation of the extracellular matrix (2, 7, 8). In particular, these two cytokines have been shown to target the catabolism of aggrecan, the major proteoglycan present in hyaline articular cartilages. Several studies have now led to a number of important discoveries, which have defined at least seven specific cleavage sites along the protein core of aggrecan (9–12). Amino acid sequence analysis of cartilage proteoglycan breakdown products has defined the two major sites of proteolytic cleavage in aggrecan, both found within the interglobular domain (IGD)1 of the aggrecan core protein. These occur between amino acid residues Asn341–Phe342 and Glu373–Ala374 (human sequence enumeration (13)). Several matrix metalloproteinases (MMP1, -2, -3, -7, -8, -9, and -13) have now been shown to cleave aggrecan at the former cleavage site. The second cleavage site was first described in the early 1990s (10–12). Now, however, almost 6 years later, the agent(s) responsible for cleavage at the Glu373–Ala374 peptide bond still remain to be identified. This uncharacterized activity, termed “aggrecanase” (12, 14), appears to have specificity for Glu-Xaa peptide bonds where Xaa is Ala, Gly, or Leu (9, 11, 15). The majority of aggrecan catabolites found in the synovial fluids of patients with arthritis appear to be generated by aggrecanase activity (15, 16). In a recent study, Fosang et al. (17) reported the presence of aggrecan catabolites in synovial fluid that contained the amino-terminal sequence FFG . . . , which indicates that matrix metalloproteinases are also involved in aggrecan catabolism.

Culture systems have been manipulated with a variety of agents that enhance proteoglycan catabolism in efforts to discover the agent(s) responsible for aggrecan breakdown. However, these studies have been unsuccessful in defining any specific agent responsible for the degradation of aggrecan (18). Nonetheless, experimental data using purified aggrecan and modified aggrecan as a substrate for testing purified enzyme preparations in vitro have yielded some information on the specific cleavage sites of the enzymes themselves, but this work has not directly helped in determining the identity or biochemical properties of aggrecanase (14, 19, 20).

In previous studies (2, 21) we have developed a number of monoclonal antibodies (neoepitope antibodies) that recognize unique proteoglycan degradation products that have been generated by specific proteinases including aggrecanase. These antibodies have proved useful as tools in studying the mechanisms of the breakdown of proteoglycan in cartilage explant culture systems. The purpose of this current work was to further refine culture systems that would facilitate the identification of the agents responsible for the cleavage of aggrecan at the 374ARGSVI . . . site in the IGD of aggrecan. In this study,

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¶ The abbreviations used are: IGD, interglobular domain; rAgg1, a recombinant substrate containing the IGD polypeptide sequence of human aggrecan; FBS, fetal bovine serum; PAGE, polyacrylamide gel electrophoresis; DMEM, Dulbecco’s modified Eagle’s medium.
**Recombinant Substrate Cleaved by Aggrecanase**

**Fig. 1. Schematic representation of the subcomponents of the rAgg1 recombinant substrate.** The recombinant substrate consists of an 8-amino acid-long FLAG epitope peptide at the amino terminus, a 127-amino acid-long IgD polypeptide (Thr331–Gly457, assuming Val20 is the native amino terminus) of the human aggrecan IGD, a 4-amino acid-long spacer peptide (Gly22), and a 232-amino acid polypeptide sequence from the human immunoglobulin hinge, CH2 and CH3 constant regions at its carboxyl terminus. Also illustrated are the nucleotide donor and acceptor sites in alternate splicing that produces a minor 39-kDa product that has 126 amino acids of the IGD and the 4-amino acid spacer deleted from the expressed protein.

We describe the development of an in vitro culture system that has enabled us to study the activity of aggrecanase against an artificial recombinant substrate in a cell culture system that is free of inherent endogenous proteoglycans and other extracellular components. The use of previously characterized neo-epitope antibodies to key cleavage sites in the IGD of aggrecan facilitated the monitoring of products generated by the proteolytic action of aggrecanase in this system.

**EXPERIMENTAL PROCEDURES**

**Materials—**Alkaline phosphatase-conjugated second antibody and substrate used in Western blot analysis were obtained from Promega as the Problot Western blot AP system (catalog no. W3920). Nitrocellulose (0.2-μm pore size) was obtained from Schleicher and Schuell. Monoclonal antibody M1, which recognizes the FLAG™ epitope, and anti-FLAG M1 affinity gel were both obtained from Eastman Kodak Co.

**Preparation of the rAgg1 Genetic Construct—**The genetic construct for rAgg1 codes for the signal sequence of the lymphocyte glycoprotein T1/Leu-1 (CD5), the 8-amino acid-long FLAG epitope (22), the 127-amino acid-long interglobular domain of the human cartilage large aggregating proteoglycan aggrecan (Thr331–Gly457, assuming Val20 is the native amino terminus (13)), a 4-amino acid-long spacer (Gly22), and a 232-amino acid polypeptide sequence from the human IgD hinge, CH2 and CH3 constant regions (see Fig. 1), thus giving rise to a secreted fusion protein with an expected molecular mass of 41,059. Aggrecan cDNA sequences encoding the interglobular domain were amplified by the reverse transcriptase polymerase chain reaction (23) with synthetic oligonucleotides complementary to sequences flanking this region. Total RNA was extracted from human cartilage tissue obtained from joint replacement surgery according to Adams et al. (24). Oligonucleotides were designed to contain additional sequence information regarding the 8-amino acid FLAG epitope and the GDPE to allow the creation of restriction enzyme cleavage sites at the 5’ and 3’ extremities of the amplified cDNA segments to facilitate subsequent insertion into the CD5-IgG1 expression vector, modified to contain a 3′ NheI site (25). The primer encoding the FLAG epitope, the amino-terminal half of the interglobular domain, and a rabbit IgG constant region including an NheI site was synthesized with the following sequence: 5′-CCG GGG GAT CTC AGA CGA CTA CAA GGA CGA CTA CAA GAC AGG TGA AGA CTT TGT GGA C. A reverse primer encoding the carboxyl-terminal end of the interglobular domain, the GDPE spacer, and a splice donor site containing a BamHI site had the sequence: 5′-CGG GGG GGA TCC CTC CTC CCT GGC AAA TGC GGC TGC CC. The polymerase chain reaction product was digested with NheI and BamHI and ligated to a NheI and BamHI cut vector CD5-IgG1 (25).

**Production and Purification of rAgg1 in COS Cells—**The genetic construct was transfected into COS cells via DEAE-dextran (26). Cells grown in DMEM containing 10% FBS were trypanosized, seeded onto freshly prepared 12-mm Transwell filters, and allowed to adhere for 5 days. On the 3rd day, fresh medium containing 10% FBS was added. Supernatants were harvested, centrifuged to remove nonadherent cells and debris, pooled, and stored at 4°C. Fusion proteins were affinity-purified via anti-FLAG M1 affinity gel (Kodak) according to the manufacturer’s protocol. The yield was 1–3 μg of fusion protein/ml of culture supernatant.

**Culture of Rat Chondrosarcoma Cells and the Recombinant IGD Substrate (rAgg1) in Agarose in the Presence or Absence of Retinoic Acid—**Rat chondrosarcoma cells were plated in 75-cm² flasks and maintained in DMEM medium containing 5% FBS and 50 μg/ml gentamicin. Confluent monolayers in 75-cm² flasks were harvested by trypsinization (0.25% trypsin (w/v) in DMEM containing 0.4 mM EDTA) for 15–20 min at 37°C with agitation followed by digestion in 0.05% collagenase (w/v) in DMEM for 1–2 h. The cells were then resuspended in DMEM at 4 × 10⁶ cells/ml. 24-well culture plates were coated (200 μl/well) with a 1% (w/v) solution of FMC Seaplaque-agarose in DMEM. The agarose was solidified by incubation at 4°C for 30 min (27) and then re-equilibrated to 37°C. The chondrocyte cell suspension (described above) was diluted with a 2% (w/v) solution of Seaplaque-agarose in DMEM such that the final cell concentration was 2 × 10⁵ cells/ml. Aliquots (200 μl) of the agarose cell suspension containing 0.4 × 10⁵ cells were layered over the previously prepared agarose plugs. Immediately following this, 50 μg of rAgg1 was added to the appropriate wells and mixed by agitation. The plates were then incubated at 4°C for 15 min to solidify the agarose and re-equilibrated at 37°C. Experimental medium (with or without retinoic acid) was then added to triplicate wells containing the recombinant IGD construct, as follows: control cultures, DMEM plus gentamicin (50 μg/ml) and treated cultures, DMEM plus gentamicin (50 μg/ml) plus 10⁻⁶ M retinoic acid (2). Agarose cell cultures were then maintained at 37°C in 5% CO₂ for 96 h after which media and agarose cell matrix extracts were further analyzed.

**Culture of Rat Chondrosarcoma Cells in Agarose in the Presence of Medium Containing rAgg1 Substrate in the Presence or Absence of Retinoic Acid—**Rat chondrosarcoma cells (i.e., no rAgg1) were set up as described above. Experimental medium (with or without retinoic acid) containing 20 μg of rAgg1 was added to duplicate cultures. Thus, in this experiment the chondrocytes were exposed to the multimolecule aggregating rAgg1 substrate in the medium. Agarose cultures were maintained as described above, and media were harvested at 16, 24, 48, 72, and 96 h for further analysis as described below.

**Culture of Bovine Chondrocytes in Agarose in the Presence of Medium Containing rAgg1 Substrate in the Presence or Absence of Retinoic Acid—**Bovine articular chondrocytes were isolated from the metacarpophalangeal joints of 2–3-year-old cows using established procedures (28, 29). Briefly cartilage was removed from the joint surfaces under sterile conditions. The tissue was digested in 0.1% Pronase in DMEM containing 5% FBS for 1 h at 37°C with agitation, washed, and further digested with 0.04% collagenase in DMEM containing 5% FBS overnight at 37°C with agitation. Cells were then filtered through a 0.2-μm Nitex filter and washed before cell numbers were established. Cells were then plated in agarose (without substrate) as described above for the rat chondrosarcoma cells. Experimental medium (with or without retinoic acid) containing 20 μg of rAgg1 was added to duplicate cultures. Bovine agarose cultures were maintained as described above and media were harvested at 24, 48, 72, and 120 h for further analysis as described below. In a specific experiment conditioned media samples were obtained from control and retinoic acid-treated cultures at 120 h; these were used to demonstrate the soluble nature of aggrecanase activity in retinoic acid-treated cultures.

**Analysis of Experimental Media from Control and Retinoic Acid-stimulated Agarose Cultures—**Media (96 h) from cultures where both the cells and the rAgg1 were embedded in agarose were dialyzed exhaustively against distilled HzO, lyophilized, and reconstituted in an equal volume of SDS-PAGE running buffer containing mercaptethanol. Samples were subjected to SDS-PAGE, transferred to nitrocellulose, and immunolocated with either polyclonal or monoclonal antibodies using procedures described below. Media from cultures containing only rAgg1 (with or without retinoic acid) from the cells embedded in agarose were dialyzed exhaustively against distilled HzO, lyophilized, and reconstituted in SDS-PAGE sample buffer (at a
concentration of 1 μg/μl rAgg1 containing 10% (v/v) mercaptoethanol. Samples were subjected to SDS-PAGE and immunolocation as described below.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analyses—Samples containing native recombinant IGD substrate and its catabolites were electrophoresed on 10% polyacrylamide slab gels in SDS using procedures described by Laemmli (30). After electrophoresis, the fractionated proteins were electrophoretically transferred to nitrocellulose and immunolocalized with a 1:1000 dilution of anti-FLAG monoclonal antibody M1 or monoclonal antibody BC-3 (anti-ARG . . . ) and a subsequent alkaline phosphatase-conjugated second antibody using procedures previously described (2). In general, the immunoblots were incubated with substrate for 5–15 min at room temperature to achieve optimum color development.

BC-3 Antibody Specificity—For experiments establishing antibody BC-3 specificity duplicate lanes containing 1 μg of rAgg substrate and product generated from cultures exposed to retinoic acid for 96 h were subjected to SDS-PAGE and transferred to nitrocellulose. The lanes were separated and one replicate was immunolocalized with antibody BC-3 (1:10,000) and the second replicate was immunolocalized with BC-3 antibody (1:10,000) that had been preincubated for 1 h at 37 °C with 2.8 mg/ml synthetic peptide containing the amino-terminal sequence corresponding to the amino terminus of the IGD (31). This peptide consists of the amino acids RAVAKVD... (where the indicated X residue was 25% random). No reactivity was observed with BC-3 recognizing an epitope in the amino terminus of the IGD. In addition, we have used MALDI (matrix assisted laser desorption ionization) spectroscopy to obtain a more accurate molecular weight of the reduced 72-kDa rAgg1 substrate. These results indicate a molecular mass of approximately 55 kDa (data not shown). Collectively, these data more closely reflect the calculated molecular weight and indicate that the 72-kDa isofrom has an anomalous migration in SDS-PAGE analyses.

Immunohistochemical Analysis of Recombinant IGD Metabolites from Agarose Cultures Containing rAgg1 Treated with and without Retinoic Acid—rAgg1 was immobilized in agarose cell cultures containing rat chondrosarcoma chondrocytes and maintained in medium with or without retinoic acid for 96 h. SDS-PAGE and Western blot analysis of rAgg1 and its metabolites released into the culture media are shown in Fig. 3.

Immunochemical staining with anti-FLAG monoclonal antibody M1 (recognizing an epitope in the FLAG domain at the amino terminus of the recombinant substrate) showed no differences in the pattern of rAgg1 metabolites present in medium from either control cultures or retinoic acid-treated cultures (Fig. 3, lanes 1 and 2). However, immunolocation with monoclonal antibody BC-3 (anti-ARG . . . ) showed positive reactivity only in the retinoic acid-treated culture medium where it reacted with a band migrating at approximately 66 kDa that was not recognized by the anti-FLAG monoclonal antibody M1 (Fig. 3, lane 4, lower arrow). Immunochemical staining with BC-3 showed no reactivity in control cultures without retinoic acid treatment (Fig. 3, lane 3). This result indicates that aggrecanase catabolism of the 72-kDa form of rAgg1 has occurred through the removal of a small peptide (6–10 kDa), containing the FLAG epitope from the amino terminus of the rAgg1 construct. As expected, there was no-terminal amino acid sequence analysis indicated that the 72-kDa form consisted of the correct FLAG and IGD peptide sequence. However, amino acid sequence analysis of the 39-kDa form gave the sequence DYKKD... (data not shown), indicating that the IGD sequence had been deleted (see Fig. 1). A disparity occurs in the calculated molecular mass and the observed SDS-PAGE migration of the 72-kDa isofrom of reduced rAgg1 that contains the IGD. We have performed enzymatic deglycosylation analyses that indicate the presence of both O- and N-glycosylation amounting to approximately 10 kDa (data not shown). In addition, we have also used MALDI (matrix assisted laser desorption ionization) spectroscopy to obtain a more accurate molecular weight of the reduced 72-kDa rAgg1 substrate. These results indicate a molecular mass of approximately 55 kDa (data not shown). Collectively, these data more closely reflect the calculated molecular weight and indicate that the 72-kDa isofrom has an anomalous migration in SDS-PAGE analyses.

RESULTS

Characteristics of the Recombinant IGD Construct—Transfection of the recombinant IGD construct into COS-7 cells resulted in the cellular expression and subsequent secretion of the recombinant IGD product into the medium. The use of an anti-FLAG M1 affinity gel facilitated its purification from the transfected cell medium. One liter of transfected COS cell medium yielded 1–3 mg of rAgg substrate and product generated from cultures exposed to retinoic acid for 96 h were subjected to SDS-PAGE and transferred to nitrocellulose. The lanes were separated and one replicate was immunolocalized with antibody BC-3 (1:10,000) alone, and the second replicate was immunolocalized with BC-3 antibody (1:10,000) that had been preincubated for 1 h at 37 °C with 2.8 mg/ml synthetic peptide containing the amino-terminal sequence ARGLV..., that was prepared with free amino and carboxy termini on an Applied Biosystems 431A automated synthesizer using FMoc (N-(9-fluorenyl)methoxycarbonyl) chemistry with the peptide purified on a C8 reverse-phase column, and the structure was confirmed by electrospray mass spectrometry and amino acid analysis (Advanced Biotechnology Center, Charing Cross and Westminster Medical School). Second antibody and substrate development were as described above.

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no evidence that the 39-kDa isoform had been catabolized since it did not contain the IGD sequence.

Immunoblot analyses with antibody BC-3 were also performed on media obtained from cultures with and without retinoic acid exposure where no rAgg1 was added. No glycosaminoglycan was detected in the medium from these cultures. In addition, Western blot analysis of the medium showed no immunostaining with antibody BC-3. This indicates that insufficient amounts of endogenous rat aggrecan were synthesized by the cells maintained in serum-free culture for 4 days for it to act as an endogenous substrate for aggrecanase activity (results not shown). This conclusion is also supported by the observation that retinoic acid essentially completely inhibits aggrecanase synthesis in monolayer cultures (3).

**Time Course of Release of Aggrecanase-generated Catabolites from rAgg1 Using Rat Chondrosarcoma Cultures**—In a subsequent experiment we used this culture system to investigate whether retinoic acid-induced aggrecanase activity could diffuse from the agarose containing the cells and catabolize rAgg1 sequestered in the medium (probably because of its large aggregate properties). For these experiments, the chondrocytes were suspended in agarose alone. The rAgg1 substrate was added to control or retinoic acid-containing medium, and culture media were harvested at 16, 24, 48, 72, and 96 h. Thus, to catabolize the rAgg1 aggregates the aggrecanase activity would have to diffuse through the agarose and into the medium. The results of immunolocation analyses of the rAgg1 medium metabolites with antibody BC-3 are shown in Fig. 4A. These results indicate that aggrecanase-generated products occur after 48 h of retinoic acid exposure (Fig. 4A, lanes 4–6). At 72 and 96 h a BC-3 positive slower migrating band of approximately 121 kDa was evident. This band probably represents a minor aggrecanase product of aggregated rAgg1 that results from incomplete reduction. Data supporting this conclusion are indicated in Fig. 4C where only 1 μg of rAgg1 (96 h) was loaded, and this minor component was not detected. No evidence of aggrecanase activity was evident in control cultures after 96 h (Fig. 4, A and C, lane 1). These results suggest that aggrecanase can occur as a non-cell-associated proteolytic activity that is present in the conditioned medium.

Additional experiments were also performed to discriminate better the undigested substrate from the 66-kDa aggrecanase-generated product. Fig. 4B shows immunolocation analysis of rAgg1 medium metabolites (1 μg) from cultures exposed to retinoic acid for 96 h (Fig. 4B, lane 1) that was subsequently probed with anti-FLAG M1 monoclonal antibody (Fig. 4B, lane 2) to identify undigested substrate containing the FLAG sequence at the amino terminus. These results clearly show the molecular mass shift from the 72-kDa undigested rAgg1 isoform to the 66-kDa aggrecanase-generated BC-3 positive product.

In addition, we also performed experiments to verify the specificity of the BC-3 antibody (Fig. 4C). Lane 1 of Fig. 4C shows BC-3 positive immunoreactivity on rAgg1 metabolites (1 μg) present in medium from cultures treated with retinoic acid for 96 h. However, this BC-3 reactivity is completely removed by preincubation of the antibody with 2.8 mg/ml synthetic peptide containing the amino-terminal sequence ARGSVI . . . . . .

**Investigation of Aggrecanase Catabolism of rAgg1 Using Freshly Isolated Bovine Chondrocytes in Agarose Culture**—Similar experiments with freshly isolated bovine articular chondrocytes substituting for Rx cells in agarose culture with and without retinoic acid exposure were also performed. The results of immunolocation analyses of media with antibody BC-3 at 24, 48, 72, 96, and 120 h of culture are shown in Fig. 5. The results indicate that for bovine chondrocytes rAgg1 catabolites produced by aggrecanase are evident between 96 and 120 h of culture in the presence of retinoic acid. The result also suggests that aggrecanase activity is diffusible and takes approximately 48–72 h longer to be expressed in bovine chondrocytes compared with the Rx rat chondrosarcoma cell cultures (Fig. 4, lanes 5 and 6).

An experiment was performed to demonstrate the diffusible properties of aggrecanase. Conditioned medium (120 h) from both control and retinoic acid-treated bovine cultures were collected and centrifuged to remove any insoluble components,
and 1 ml of the supernatant was incubated with 5 μg of rAgg1 for 16 h at 37 °C. Replicate samples without rAgg1 substrate were treated similarly (as controls). After incubation, the samples were processed as described under “Experimental Procedures,” and the Western blots were immunolocalized with antibody BC-3 (Fig. 6). No immunopositive BC-3 reactivity was seen in the control medium (Fig. 6, lane 1) or in media that were incubated without rAgg1 (results not shown). However, medium from retinoic acid-treated cultures showed strong BC-3 reactivity (Fig. 6, lane 2) indicating that aggrecanase was a soluble proteolytic activity present in the conditioned medium.

**DISCUSSION**

These results are the first report of the production and use of a recombinant protein substrate for the study of aggrecanase activity. In addition, we have developed a novel cell culture system to monitor aggrecanase activity without the complication of endogenous aggrecan acting as a substrate. The unique aggregating properties of this artificial substrate (rAgg1) facilitated its immobilization in the agarose cell culture system and also its sequestration in the medium compartment for other studies demonstrating the diffusible properties of this activity. Furthermore, we were able to use freshly isolated chondrocytes without the need to establish an endogenous extracellular matrix to act as a substrate for monitoring aggrecanase activity. The results obtained with the recombinant IGD construct demonstrate that there is not a need for a G1 and/or a G2 domain on the IGD of aggrecan for cleavage at the aggrecanase site. The rAgg1 construct showed no positive staining on Western blot analysis with an anti-keratan sulfate antibody 5-D-4 (results not shown). Therefore, there appears to be no requirement for keratan sulfate chains within the IGD that may give rigidity to this region in the native aggrecan molecule.

The induction of aggrecanase activity in rat chondrosarcoma cells (2, 3) and bovine monolayer cultures (3) has been described previously. Our studies demonstrate that aggrecanase activity is only expressed several hours after the chondrocytes are exposed to retinoic acid. Interestingly, the same time period (48 h) is required for aggrecanase induction from both rat and bovine chondrosarcoma monolayer cell cultures (3) and the same cells maintained in agarose cultures (this study). This finding indicates that the presence of the agarose matrix is not impeding the diffusion of aggrecanase into the conditioned medium. This suggests that time may be required for synthesis and processing of the proteolytic activity. Alternatively, expression of aggrecanase activity may require time for the involvement of additional enzyme cascades to produce the active form(s) of the enzyme. This time-dependent expression of aggrecanase was demonstrated using two different animal sources of chondrocyte cultures subjected to SDS-PAGE and Western blotting. Aggrecanase-generated rAgg1 catabolites were visualized using monoclonal antibody BC-3. C, control.

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