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Abbreviations used: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; AU, arbitrary units; CS, chondroitin sulfate region; dw, dry weight; sGAG, sulfated glycosaminoglycan; Gu-PG, guanidinium extracted proteoglycan; IGD, interglobular domain; KS, keratan sulfate region; MMP, matrix metalloproteinase; Mw, molecular weight; ND, not determined; OA, osteoarthritis; SD, standard deviation; SF, synovial fluid; std, standards; ww, wet weight.

SYNOPSIS

Mature aggrecan is generally C-terminally truncated at several sites in the chondroitin sulfate (CS) region. Aggrecanases and matrix metalloproteinases (MMPs) have been suggested to be responsible for this digestion. To identify if calpain, a common intracellular protease, has a specific role in the proteolysis of aggrecan we developed neoepitope antibodies (PGVA, GDSL and EDLS) against calpain cleavage sites and used Western blot to identify calpain generated fragments in normal and osteoarthritis (OA) knee cartilage and synovial fluid (SF) samples. Our results showed that human aggrecan contains six calpain cleavage sites: one in the inter globular domain (IGD), one in the keratan sulfate (KS) region, two in the CS1 and two in the CS2 region. Kinetic studies of calpain proteolysis against aggrecan showed that the aggrecan molecule was cleaved in a specific order where cuts in CS1 was the most and cuts in KS was the second most preferred cleavage. OA and normal cartilage contained low amounts of a calpain generated G1-PGVA fragment (0.5-2%) compared to aggrecanase, G1-TEGE (71-76%), and MMP generated, G1-IPEN (23-29%), fragments. Significant amounts of calpain generated GDLS and EDLS fragments were found in OA and normal cartilage, and a ARGS-EDLS fragment was detected in arthritic SF samples. These results indicate that calpains are involved in the C-terminal truncation of aggrecan and might have a minor role in arthritic diseases.

INTRODUCTION

Aggrecan is the major extracellular proteoglycan in articular cartilage, and together with type II collagen it provides the tissue with mechanical properties of reversible compressibility. Degradation of the extracellular matrix is a hallmark in arthritic diseases and in joint injuries, and one of the first proteins to be degraded is aggrecan [1-2]. The main proteases responsible of aggrecan degradation in arthritis and joint injuries are the aggrecanases (ADAMTS4 and ADAMTS5, a disintegrin and metalloproteinase with thrombospondin motifs) and the MMPs (MMP 1-3, 8, 9, 13) [3-5]. Cathepsins and calpains have also proteolytic activities against aggrecan [6-11], although their role in aggrecan degradation is not totally understood.

Calpains are calcium dependent cysteine proteinases with multiple cellular functions, and calpastatin is their endogenous inhibitor [12]. There are two major forms of calpains, μ-calpain (calpain 1) and m-calpain (calpain 2), and they are activated by micro- and milli-molar concentrations of Ca^{2+}, respectively. Calpains are classified as intracellular proteases [12-15], but calpains have also been found in the synovial fluid [16-18] and in the culture medium of arthritic cartilage explants [19] and chondrocytes [20], suggesting that calpains might have a role in the turnover or degradation of the cartilage extra cellular matrix.

In the process of learning more about cartilage degradation, analyzing the proteolysis of one of the major key matrix proteins – aggrecan, we here investigate the possible role of calpain digestion of human aggrecan looking at preferred order of cleavage and analyzing the *in vivo* content of calpain generated aggrecan fragments in knee cartilage and synovial fluid comparing these with fragments generated from the major aggrecanolysis enzymes of aggrecanases and MMPs.

EXPERIMENTAL

Cartilage and synovial fluid aggrecan

Human OA-cartilage pool (n = 10) and synovial fluid OA pool (n = 47) was made as described previously [23]. Knee patella cartilage (normal cartilage with no records of knee disease or injury) was obtained from a newborn subject and from a youth (14 years old) subject. Preparation of cartilage proteoglycan guanidinium extraction (Gu-PG) and A1D1-D3 fractions, and SF D1-D3 and A1-A3 fractions was as described previously [21]. Samples were stored at –80°C. Sulfated glycosaminoglycan (sGAG; Alcian blue precipitation) analysis of samples was conducted as described previously [5,22]. All patient related procedures were approved by the ethics review committee of the Medical Faculty of Lund University.

Calpain *in vitro* proteolysis of aggrecan

Human aggrecan (A1D1 fraction) was digested by m-calpain (porcine kidney m-calpain; EC nr 3.4.22.17, Calbiochem) using four different conditions: 1:686, 1:1956, 1:6976 and 1:20930 (μg enzyme/μg aggrecan dry weight) corresponding to following mol enzyme (Mw = 109 kDa) per mol substrate (Mw = 1500 kDa) ratios; 1:50, 1:142 (standard digestion), 1:507 and 1:1521. Digestion started when m-calpain was added to the aggrecan, solved in calpain digestion buffer (110 mM Imidazole, 5 mM 2-mercaptoethanol, 1 mM EGTA, 7 mM $CaCl_2$, pH 7.5; final concentrations), and was incubated for up to 2h at 30°C. Aliquots were taken from the sample at different time points and the reactions were stopped by adding an excess of EDTA. The samples were diluted with Na- and Tris-acetate solutions (50 mM Na-acetate, 50 mM Tris-acetate, 35 mM Imidazole, 1.6 mM 2-mercaptoethanol, 10 mM EDTA, pH 7.5;

final concentrations) and deglycosylated with chondroitinase ABC, keratanase and keratanase II as previously described [5].

Deglycosylation before calpain *in vitro* digestion was conducted as follows: Human aggrecan (A1D1 fraction) was incubated with (+ *sample*) or without (- *sample*) deglycosylation enzymes at 37°C as previously [5]. The samples were then dialyzed (10 000 Da cut off) against calpain digestion buffer, and thereafter calpain *in vitro* digested for 2h (standard digestion), and stopped by EDTA addition. The - *sample* was then incubated with deglycosylation enzymes while the + *sample* was incubated without enzymes.

Aggrecan antibodies and Western blot

Monoclonal anti- IGD antibody (6-B-4), recognizing the IGD sequence EPEEPFTFAPEI was from Abcam (Cambridge, UK). Neo-specific polyclonal anti-KEEE, anti-TEGE and anti-IPEN sera were from Merck [5,23]. Polyclonal AVPV, EDLS, GDLS, GLPS, GVPS and PGVA antiserum were made (Innovagen, Lund Sweden) by injecting into rabbits (one peptide/rabbit) the following immunogen peptides: AVPVEEETTAC, SGVEDLS, CSGVGDLS, GLPSGC, GVPSGC and CKKIVTQVVPGVA where the N- or C-terminal cysteins were conjugated to Keyhole Limpet Hemocyanin. Sera was collected at day 42 (1st bleed sera, used herein), 63 (2nd) and at day 84 (3rd bleed). Polyclonal neoepitope SELE antiserum and monoclonal ARGS antibody (OA-1) was from GlaxoSmithKline [5,24]. Polyclonal anti-G1 and anti-G3 antibodies were from Affinity BioReagents (Golden, CO., USA) and anti-G1 serum was a kind gift from Dr. John Sandy (Department of Biochemistry, Rush University Medical Center, Chicago). The specificity of the G1, IGD and G3 antibodies was confirmed by complete blocking of their immunoreactions in Western blot by the immunogen peptides CATEGQVRVNSIYQKVSL (G1), CDGHPMQFENWRPNQPDN (G3) and EPEEPFTFAPEI (IGD) (data not shown).

Amino acid sequences: All human aggrecan amino acid numbers in this paper are based on full length sequences starting with the N-terminal [1]MTTL- (aggrecan NCBI accession nr P16112).

Deglycosylated samples and Molecular weight (Mw) markers (Precision Plus Protein Standards, BioRad) were separated on 3-8% or 4-12 % SDS mini-gels as previously [5]. Proteins were electrophoretically transferred onto PVDF membranes (BioRad), probed with antibodies and incubated with ECL Plus (GE Healthcare) or ECL SuperSignal West Femto (Thermo Scientific) [5]. The aggrecan fragments were visualized by using film (Amersham Hyperfilm ECL), or by using a luminescence image analyzer (Fuji Film LAS-1000) and a Multi Gauge v3.2 (Fuji Film) software. The dilutions of antibodies and antisera were as follows: G1 (1:400 for antibody, 1:5000 for sera), G3 (1:500), ARGS (1:4000), IGD (1:400), PGVA (1:10 000), EDLS (1:1000), GDLS (1:5000), SELE (1:2000), KEEE (1:1000), peroxidase conjugated goat anti-mouse IgG (10 and 20 ng/ml) and peroxidase conjugated goat anti-rabbit IgG (2.5-40 ng/ml).

Quantification of aggrecan fragments by Western blot

G1-TEGE (ADAMTS generated), G1-IPEN (MMP generated) and G1-PGVA (calpain generated) standards were made by maximum digestion of aggrecan (using A1D1 prepared from the cartilage OA-pool, Mw = 1500 kDa) with recombinant human ADAMTS-4 (24h at

37°C, 41.7 mol substrate per mol enzyme [ADAMTS-4 Mw = 62.5 kDa]) or recombinant human MMP-3 (16h at 37°C, 2.9 mol substrate per mol enzyme [MMP-3 Mw = 42.8 kDa]) in proteinase digestion buffer [5], or with m-calpain (2h at 30°C, standard condition). The standards were deglycosylated and different amounts (6 or 9 standard points) were loaded on gels. Standards and samples were visualized and quantified using the image analyzer with software, and from the linear range of standard curves mol quantities were calculated for each fragment. The evaluation of Western blot quantification of aggrecan fragments has been described previously [25]. For kinetic studies the aggrecan fragments were quantified similarly, although without standards, expressing the amounts in arbitrary units (AU). Data is presented as mean with standard deviations (SD).

RESULTS

Neoepitope antibodies against calpain cleavage sites in aggrecan

To study calpain proteolysis of aggrecan we produced neoepitope antibodies against following human aggrecan sequences: -IVTQVVPGVA and AVPVEEETTA- corresponding to a calpain cleavage site PGVA$^{709}\downarrow^{710}$AVPV in the KS region; -SGVGDLS, -SGVEDLS, GLPSG- and GVPSG- corresponding to following calpain cleavage sites in the CS1 region GDLS$^{954}\downarrow^{955}$GLPS, GDLS$^{973}\downarrow^{974}$GLPS, GDLS$^{1353}\downarrow^{1354}$GLPS, EDLS$^{1411}\downarrow^{1412}$RLPS and GDLS$^{1431}\downarrow^{1432}$GVPS. Specificity of the antibodies was determined by using immunogen peptides, spanning peptides and a dummy peptide in Western blot experiments (Table 1). Neoepitope specificity of the GDLS and PGVA antibodies was confirmed by complete blocking of the immunoreactions by their immunogen peptides and by no blocking using spanning peptides or the dummy peptide (Table 1). Unfortunately, the AVPV and EDLS antibodies not only recognized their corresponding neoepitope sequence but also their internal sequences (Table 1). The specificity of the GLPSG and GVPSG antibodies could not be confirmed and was therefore not used further (data not shown).

These data suggests that the AVPV, PGVA, EDLS and GLDS antibodies recognize their corresponding immunogen sequence, although the AVPV and EDLS antibodies recognizes also the internal sequences.

The aggrecan fragments detected herein were either verified by antibodies and blocking experiments as described above or by estimation using a calculation model described previously [26].

Aggrecan as substrate for calpain proteolysis

We analyzed the human OA-pool cartilage aggrecan (A1D1 fraction) as a potential substrate for m-calpain *in vitro* digestions. The Western blots showed that the A1D1 fraction contains the following aggrecan fragments as potential substrates: G1-G3 (> 400 kDa, full length monomer); 350-370 kDa G1-KEEE^{1733}, 340 kDa G1-SELE^{1564}, 323 kDa G1-EDLS^{1411} and 210-245 kDa G1-CS1 (Figure 1A). Quantitative G1 Western blot analysis showed that the G1-KEEE, G1-SELE, G1-EDLS and G1-CS1 fragments constituted together the major part of this substrate (61%), while only 39% of the total G1 fragments were G1-G3 monomers (Figure 1B). Even though the human aggrecan prepared from OA cartilage are not mainly full length monomers these data together with data below indicates that the human OA aggrecan A1D1 fraction contains enough aggrecan fragments suitable as substrates for calpain *in vitro* studies.

Calpain *in vitro* digestion of human aggrecan

Human cartilage A1D1 aggrecan was *in vitro* digested by m-calpain and analyzed with Western blot (Figure 2). After 2h *in vitro* proteolysis several of the high Mw (> 200 kDa) G1 fragments in the origin sample were processed to smaller fragments: G1-PGVA^{709} (137 kDa), ^{366}GEED-PGVA^{709} (79 kDa) and G1-FGVG^{365} (53 kDa) (Figure 2A). Also, this digest produced two GDLS fragments with N-terminals confirmed by the AVPV antibody (data not shown), and these fragments were estimated by the calculation model as: 225 kDa ^{710}AVPV-GDLS^{1431} and 98 kDa ^{710}AVPV-GDLS$^{954/973}$ (Figure 2B). The calpain digest also generated three G3 fragments with N-terminals in the CS regions: 240 kDa CS1-G3, 204 kDa CS2-G3 and 174 kDa CS2-G3 (i.e. ^{1682}GQPS-G3 fragment) (Figure 2C). Interesting, the calpain generated G3-fragment pattern was similar (although shifted) to a aggrecanase generated G3 fragment pattern (Figure 2C), indicating that calpain cleaves aggrecan close to the aggrecanase sites in the CS2 region (Figure 3).

Comparing the *in vitro* generated fragments detected by Western blot with analysis of the human aggrecan amino acid sequence suggests the presence of six calpain cleavage sites or regions (called a-f) in the human aggrecan molecule (Figure 3): site-a at FGVG^{365}↓^{366}GEED in IGD; site-b at PGVA^{709}↓^{710}AVPV in KS; region-c at GDLS^{954}↓^{955}GLPS and at GDLS^{973}↓^{974}GLPS in CS1; region-d at GDLS^{1353}↓^{1354}GLPS, EDLS^{1411}↓^{1412}RLPS, GDLS^{1431}↓^{1432}GVPS, TDLS^{1452}↓^{1453}GLPS and at EDLS^{1472}↓^{1473}GLPS in CS1; site-e at cleavage between ^{1573}GAGE-^{1597}GTEL in CS2; site-f at PDLS^{1681}↓^{1682}GQPS in CS2.

Time dependent calpain digestion of aggrecan

Time dependent *in vitro* proteolysis of aggrecan by m-calpain showed fast degradation of the G1-G3 substrate (reduced by 50% after approximately 10 s), and at the same time the G1-PGVA fragment was produced (Supplementary Figure S1A). The G1-PGVA fragment was the most dominant calpain generated G1 product, and at maximum levels (after 10-15 min digestion) this fragment was approximately 9.5 times higher in concentration compared to the G1-FGVG fragment (Supplementary Figure S1B). The 240 kDa CS1-G3 fragment was the most dominant calpain generated G3 product, and at maximum production levels (after 15-30 min digestion) this fragment had approximately 5 times higher amounts than the 204 kDa CS2-G3 and 174 kDa GQPS-G3 fragments (Supplementary S1C). Higher or lower enzyme to substrate ratios compared to the standard *in vitro* digestion did not generate any new aggrecan fragments (data not shown).

Calpain cuts aggrecan in a preferred order

To determine in which order calpain cuts aggrecan we analyzed the initial kinetic speed of proteolytic fragment production and at which time these calpain generated fragments increased with 20% compared to the start level. For the CS1-G3 (240 kDa) fragment it took only 21 s to increase the fragment level with 20%, while for the GQPS-G3 (174 kDa) it took almost 1.5 min (Figure 4). From these analyses the following preferred calpain cleavage order for aggrecan was found: 1st, multiple cleavage site-d in CS1; 2nd, cleavage site-b in KS; 3rd, cleavage site-a in IGD; 4th, cleavage site-e in CS2; 5th, cleavage site-f in CS2. Due to weak signals, it was not possible to quantify the time dependent digestion of the 98 kDa AVPV-GDLS fragment, and therefore no kinetic data on the cleavage region-c (in CS1) was obtained. Except that the 3rd (site-a in IGD) and 4th (site-e in CS2) preferred cleavage site

orders were exchanged, the same calpain cleave order of aggrecan was observed in normal cartilage aggrecan (A1D1) prepared from a young subject as for aggrecan from the OA-pool (data not shown).

Deglycosylation of aggrecan before calpain addition repressed the calpain *in vitro* digestion in the KS and CS regions compared to the control, which was observed as approximately 90, 100 and 75-100% inhibition of the calpain generated PGVA, GDLS and G3 fragments, respectively (data not shown). Also, acetone precipitation of aggrecan prior calpain addition completely abolished calpain digestion (data not shown).

These data suggests that calpain has a preferred order of cleavage of aggrecan, and since the aggrecan sGAG substitutions most likely vary between mature (OA) and youth cartilage, this sGAG variation is not enough to affect the calpain cleavage in aggrecan.

Calpain generated aggrecan fragments *in vivo*

The 79 kDa GEED-PGVA fragment was not detected *in vivo* in cartilage, and could only be generated, by calpain digestion, in low concentrations *in vitro* (Figure 2A). On the other hand, accordance with previous data [10,11], the 137 kDa G1-PGVA fragment was mainly detected in the middle to low density (1.5 - 1.4 g/ml) A1D2 and A1D3 fractions from OA and normal (youth and newborn) human cartilage (Figure 5A), suggesting that only few sGAG chains are present on the G1-PGVA fragment [11]. The OA cartilage also contained a larger G1-PGVA fragment (176 kDa) (Figure 5A), and a similar G1-PGVA fragment (i.e. 160 kDa) has previously also been reported for bovine aggrecan [10]. This fragment might have different sGAG substitutions or has a crosslink to another macromolecule which the smaller 137 kDa PGVA fragment lacks. In the medium of human cartilage explants the 137 kDa G1-PGVA fragment was detected in approximately equal amounts when cultured in the presence or absence of cytokines (Figure 5A). The amount of G1-PGVA fragments in cartilage was further compared with concentrations of G1-IPEN (MMP generated) and G1-TEGE (aggrecanase generated) fragments (Supplementary Figure S2). The Gu-PG sample from the cartilage OA-pool contained (relative amounts) 76% G1-TEGE, 23% G1-IPEN and 1% G1-PGVA fragments (Table 2), and similar distribution was found in the A1D3 fractions of OA and normal cartilage (Table 2). The major part of the sGAG containing aggrecan is lost when calpain cuts in the IGD and KS regions and, therefore it is considered as pathological. Taken this into account, these data suggest that in aggrecan degradation (i.e. normal and end stage OA cartilage) the contribution by calpain digestion in the KS region is very low as compared to the digestions in the IGD made by aggrecanases and MMPs.

The 98 kDa AVPV-GDLS fragment was not detected *in vivo* in cartilage and could only be generated in very low concentrations by *in vitro* calpain digestion (Figure 2B). On the other hand, the 225 kDa AVPV-GDLS^{1431} fragment was detected (mainly in the A1D2 fractions) *in vivo* in OA and normal youth cartilage (Figure 5B), but also in A1D1 and A1D2/D3 fractions prepared from newborn cartilage (data not shown). The GDLS fragments were not detected in the cartilage explant medium (data not shown).

G1-EDLS (323 kDa) and G1-EDLS-CS2 (387 kDa) fragments were detected *in vivo* in both OA and normal (youth and newborn) cartilage (Figure 5C), and in approximately equal amounts in the cartilage explant media when cultured in presence or absence of cytokines (data not shown).

ARGS-EDLS (280 kDa) and ARGS-EDLS-SELE (300 kDa) fragments were detected, in approximately similar amounts, in SF D1 fractions prepared from acute inflammatory arthritis patients, but only the ARGS-EDLS-SELE fragment was found in the SF D1 fraction of the OA-pool (Figure 6). The PGVA and GDLS epitopes were not found in SF OA-pool fractions (i.e. D1-3 and A1-3) or in the SF D1 fractions from patients (described in [25]) with arthritis or joint injuries (data not shown).

These data suggests that calpain digestion in the CS region of aggrecan, resulting in EDLS and GDLS C-terminal fragments, is present in significant amounts in normal and OA cartilage, while the EDLS fragment is also present in some arthritic synovial fluids.

DISCUSSION

By using neoepitope and structural recognizing antibodies six calpain cleavage sites (a-f), whereof three sites (c, e, and f) are previously unknown, were detected in human aggrecan. The kinetics of m-calpain proteolysis of aggrecan showed a specific order of cleavage, where site-d (in CS1) was the most, and site-b (in KS) second most, preferred cleavage sites. Cuts in these sites generated PGVA, EDLS and GDLS aggrecan neoepitopes which were detected *in vivo* in human OA and normal knee cartilage, and the EDLS neoepitope was also observed in arthritic synovial fluids.

The calpain cleavage sites in human aggrecan does not resemble the conserved amino acid sequence (i.e. TWLK↓SPPP) at cleavage sites found in other proteins [28], although it has been indicated that calpain cleavage depends more on higher structural order rather than on the amino acid sequence of the polypeptide chain [12]. Removal of sGAG chains (by deglycosylation) from aggrecan, or precipitating the molecule, most likely results in structural changes compared to the native form, and these changes might be the reason for the hindrance of calpain to cut the aggrecan backbone. Similar inhibition of digestion has been observed by keratanase treatment of aggrecan which resulted in suppressed aggrecanase cut in the IGD, while the same treatment did not inhibit MMP-3 digestion [29].

Both m- and μ-calpains have been found in arthritic cartilage [18,19] and in OA synovial fluid [16,17], which suggests a role for calpains in cartilage degradation. On the other hand our results points towards that calpains also have a function in the general (non-pathological) turnover of aggrecan: For example 1) The G1-PGVA and G1-EDLS fragments were both found in equal amounts in the cartilage explant medium cultured in the presence or absence of cytokines. Similar data of non cytokine stimulated release of PGVA fragments have also been found by Western blot experiments in bovine meniscus cultures [30]. 2) The PGVA, GDLS and EDLS epitopes were present in both normal (young) and OA (mature) knee cartilage, where presence of these epitopes in young cartilage most likely indicates high aggrecan turnover during cartilage development. Similar data indicating EDLS epitopes in both normal and OA cartilage has been shown previously [31]. 3) OA cartilage (i.e. end stage) contains low amounts of the PGVA neoepitope as compared to the amount of MMP and aggrecanase generated IPEN and TEGE neoepitopes. 4) The preference of calpain to cut human aggrecan was higher in the less destructive CS1 region (i.e. site-d) than for the pathological sites in IGD and KS, and similar preference has also been shown for calpain digestion of bovine aggrecan [10].

It is not known if the calpain generated aggrecan fragments that we found *in vivo* was a result of intra or extracellular proteolysis. There are some studies suggesting that calpains cut aggrecan within the cells: (I) In chicken chondrocytes aggrecan is cut (e.g. due to missfolding) in the endoplasmatic reticulum by calpain and thereafter the aggrecan fragments are exported outside the cell [32]. (II) Immunohistochemical studies from human OA cartilage chondrocytes show that calpains and G1-PGVA fragments co-localize inside the cells [11]. The fact that calpains are found in normal and arthritic SF [16-18], in the medium of TNF-α stimulated chondrocytes [20], in synovial cell cultures [20] and in the medium of cartilage explants [19], suggest on the other hand that calpain digestions take place in the extracellular matrix. Probably some of the calpain cuts in aggrecan occur within the chondrocytes (e.g. cut in the PGVA↓AVPV site), while further calpain processing (or calpain cuts in another pool of aggrecan molecules) take place in the extracellular matrix. Further investigations using cell cultures have to be conducted to show if and where intra and/or extra cellular calpain cuts take place.

In addition to the different interpretations and differences compared to other studies, this study has some general limitations: 1) Normal cartilage was from the patella and obtained from only two subjects, and the cartilage explant media was only from one subject. 2) Any difference in the affinity of the antibodies for fragments with different sizes carrying the same epitope would affect the detected signal. 3) The relative distribution of G1-TEGE, G1-IPEN and G1-PGVA fragments in end-stage OA cartilage gives a snap shot of the accumulative events which occurred in the tissue. During this process one or more of these fragments could have been further processed, e.g. by chondrocyte endocytosis [33], which would misinterpret the contribution of the different proteases. 4) The PGVA fragments detected *in vivo* could be a result from cathepsin S digestions [34], however cathepsins are lysosomal enzymes with an acidic pH optimum while calpains have a pH optimum of 7-7.5 [35,36].

Our portfolio of antibodies, recognizing aggrecan neoepitopes generated by different proteases, is an important tool in the study of aggrecan degradation. All the aggrecan fragments discussed herein were either verified both N- and C-terminally by aggrecan neoepitope or structural antibodies, or by estimation using a calculation model [26]. C-terminal truncations of large sGAG containing aggrecan fragments, where the G3 domain and parts of the CS region is lost, has shown to be a result of aggrecanase (generating –SELE and –KEEE fragments, [3,5]) and MMP (generating –GVED, [37]) digestions, although it has also been suggested that calpains are involved in these C-terminal truncations [3,5,31]. Our results, identifying several calpain generated fragments *in vivo* (G1-EDLS, AVPV-GDLS, ARGS-EDLS), strongly supports the idea that these large C-terminally truncated aggrecan fragments are also generated by the proteolytic enzyme family of calpains, although we can not rule out that calpain digestion of aggrecan could also have a role in any stage of joint diseases or injuries.

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Table 1: Specificity of neoepitope antibodies against calpain generated aggrecan fragments

Peptides[a]	AVPV	PGVA	EDLS	GDLS
^{710}AVPVEEETTA (AVPV immunogen)	+	-	ND	ND
IVTQVVPGVA^{709} (PGVA immunogen)	-	+	ND	ND
SGVGDLS$^{954, 973}$ (GDLS immunogen)	ND	ND	-	+
SGVEDLS^{1411} (EDLS immunogen)	ND	ND	+	-
IVTQVVPGVAAVPVEEETTA^{719} (PGVAAVPV spanning)	+	-	ND	ND
SASGVEDLSRLPSG^{1416} (EDLS spanning 1)	ND	ND	+	-
SASGAEDLSGLPSG^{1477} (EDLS spanning 2)	ND	ND	-	-
SASGVGDLSGLPSG$^{959, 978}$ (GDSL spanning)	ND	ND	-	-
ASTASELE^{1564} (dummy[b])	-	-	-	-

The specificity of AVPV, PGVA, EDLS and GDLS neoepitope antibodies was determined by Western blot experiments, where antibodies were incubated (30 min at 22^{o}C) with peptides (10 µM) prior addition to PVDF membranes containing m-calpain *in vitro* digested aggrecan (A1D1). + = peptide blocked the Western blot signal. - = peptide did not block the Western blot signal. Spanning = a peptide which span the immunogen sequence. ND = not determined.

a) Peptide sequences with corresponding human aggrecan amino acid numbers.

b) Peptide dummy with a sequence with no relevance for the antibodies tested.

Table 2: Amounts of aggrecan G1-IPEN, G1-TEGE and G1-PGVA fragments in OA and normal cartilage

	OA cartilage[a]				Normal cartilage[b]	
	Gu-PG		A1D3		A1D3	
	Conc. (pmol/mg ww)	Rel.	Conc. (pmol/mg dw)	Rel.	Conc. (pmol/mg dw)	Rel.
G1-IPEN	3.38 (0.83)	22.8 (1.1)	438 (153)	28.7 (6.8)	311 (119)	26.7 (6.5)
G1-TEGE	11.25 (2.83)	75.9 (1.0)	1057 (135)	70.8 (6.8)	829 (289)	71.4 (6.1)
G1-PGVA	0.18 (0.05)	1.3 (0.2)	7 (1)	0.5 (0.05)	20 (4)	1.9 (0.6)

Samples (probed with IPEN, TEGE and PGVA antibodies) were quantified using standards made from MMP-3, ADAMTS-4 and m-calpain maximum digests of human aggrecan. Relative (Rel.) amounts (in %) were first calculated (using the linear range of the standard curves) for each Western blot (see Supplementary Figure S2) and thereafter the mean Rel. value (SD) were calculated. OA Gu-PG, n=5; OA A1D3, n=6; normal A1D3, n=3 blots. dw = dry weight cartilage. ww = wet weight cartilage.

a) Guanidinium extracted proteoglycan (Gu-PG) and A1D3 fraction prepared from the knee cartilage OA-pool.

b) A1D3 fraction prepared from a normal (knee healthy) youth cartilage sample.

Figure 1: Aggrecan G1-fragments in OA cartilage
Aggrecan (A1D1) fragments were deglycosylated and separated on 3-8% SDS-gels. (A) Western blot analysis with domain recognizing antibodies anti-G1 (Affinity BioReagents), -IGD, -G3; and with C-terminal neoepitope antibodies anti-EDLS, -SELE and -KEEE. Note that the larger EDLS band (marked 1) shows the internal immuno-signal in the G1-EDLS-G3 (>400 kDa) fragment, while the smaller EDLS band (marked 2) shows the C-terminal neoepitope of the 323 kDa G1-EDLS fragment. (B) Schematic figure of human full-length aggrecan with structural domains and regions with their amino acid numbers in brackets. Mean distribution (SD) of G1 fragments in the aggrecan A1D1 fraction, calculated as % of total G1-signal (n = 9 blots), are shown. Representative Western blot image from full size blotted gels are shown. 1-5 µg sGAG was loaded per lane.

Figure 2: Calpain and aggrecanase *in vitro* digestion of aggrecan
Aggrecan (A1D1, cartilage OA-pool) was *in vitro* digested by m-Calpain (+C) for 2h (mol enzyme/mol substrate, ratio = 1:142), or by ADAMTS-4 (+A) for 15 min as previously described [21]. The reactions were stopped by EDTA addition and deglycosylated fragments were separated on 3-8% SDS-gels. The aggrecan fragments were visualized by anti-G1 (sera) and anti-PGVA (A), anti-GDLS (B) and anti-G3 Western blots (C). Note, the GDLS strip with 5 µg sGAG had a long film exposure (B). Representative Western blot images from full size blotted gels, amount sGAG loaded per lane (below blots), and the aggrecan fragments (Mw in kDa) are shown. Lanes marked - = cartilage aggrecan A1D1. * = false-positive immunobands since they could not be blocked by their immunogen peptides.

Figure 3: Calpain and aggrecanase cleavage sites in human aggrecan
Six (*a-f*) calpain cleavage sites/regions in human aggrecan were found by alignment of aggrecan fragments and by amino acid sequence comparisons. Aggrecan fragments (Mw in kDa) produced by calpain *in vitro* digestion of cartilage A1D1 and detected by Western blot. For comparison, aggrecanase cleavage sites are shown above the aggrecan monomer.

Figure 4: Determination of calpains' preferred order of cleavage in aggrecan
Aggrecan (A1D1, cartilage OA-pool) was time dependently *in vitro* digested by m-calpain. The calpain generated fragments, representing different cleavage sites or regions, were quantitatively analyzed by Western blot (antibodies used are underlined): 53 kDa G1-FGVG, FGVG↓GEED (site-a in IGD); 137 kDa G1-PGVA, PGVA↓AVPV (site-b in KS); 240 kDa CS1-G3, DLS↓(G/R)LPS/GVPS (multiple site-d in CS1); 204 kDa CS2-G3, within GAGE-GTEL (region-e in CS2); 174 kDa CS2-G3, PDLS↓GQPS (site-f in CS2). The linear equation ($y = kx + l$) are shown for each fragment, and the differences (from 0 min) of fragment levels (relative units) are plotted against digestion time. The slope constants (k), indicating the initial proteolysis speed, and the time points where 20% of maximum levels were reached was used to determine the preferred order of cleavage. Only the linear parts ($R^2 = 0.9 – 1.0$) of fragment kinetics were used in the calculations. The data is from one study (i.e. one *in vitro* digest with following Western blots), although a second study showed the same preferred order of cleavage with similar slope constants (data not shown).

Figure 5: PGVA, GDLS and EDLS fragments in different cartilage fractions
Gu-PG and A1D1-D3 fractions were prepared from the knee cartilage OA-pool and from normal, youth and newborn, knee healthy cartilage. Media samples were harvested from human knee OA cartilage explants which had been cultured in the absence (-) or presence of IL-1 (20 ng/ml) and oncostatin M (OSM, 10 ng/ml) as previously described [27]. Deglycosylated samples were analyzed by PGVA (A), GDLS (B) and EDLS (C) Western

blots. Representative Western blot images from full size blotted gels with aggrecan fragments (Mw in kDa) are shown. To ease the comparison between explant media only parts of the blots are shown. For the cartilage fractions the amount sGAG loaded per lane are shown below the blots. For explant media samples equal amount media (from equal amount wet weight cartilage [= 1.8 mg]) was loaded. +C = m-calpain *in vitro* digested cartilage A1D1, 1 = Gu-PG, 2 = A1D1, 3 = A1D2, 4 = A1D3, 5 = A1D2/D3. Dimer = G1-PGVA dimer (A). * = false-positive immunoband since it could not be blocked by its immunogen peptide.

Figure 6: EDLS fragments in synovial fluid
Synovial fluid D1 fractions were prepared from the OA-pool and from patients with acute inflammatory arthritis (AA5 and AA6) as described previously [25]. The D1 samples were analyzed by ARGS, EDLS and SELE Western blots and compared to a ADAMTS4 *in vitro* digested cartilage A1D1 sample (marked +A). Note, the EDLS antibody recognizes both the internal and neoepitope sequence seen as two EDLS bands in the AA5 and AA6 samples. Parts of representative Western blot images are shown. Amount sGAG loaded per lane: ARGS, 1 µg; EDLS, 1 µg; SELE, 2 µg. SELE band-1, G1-SELE (340 kDa); band-2, IGD-SELE (275 kDa).

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Figure 1

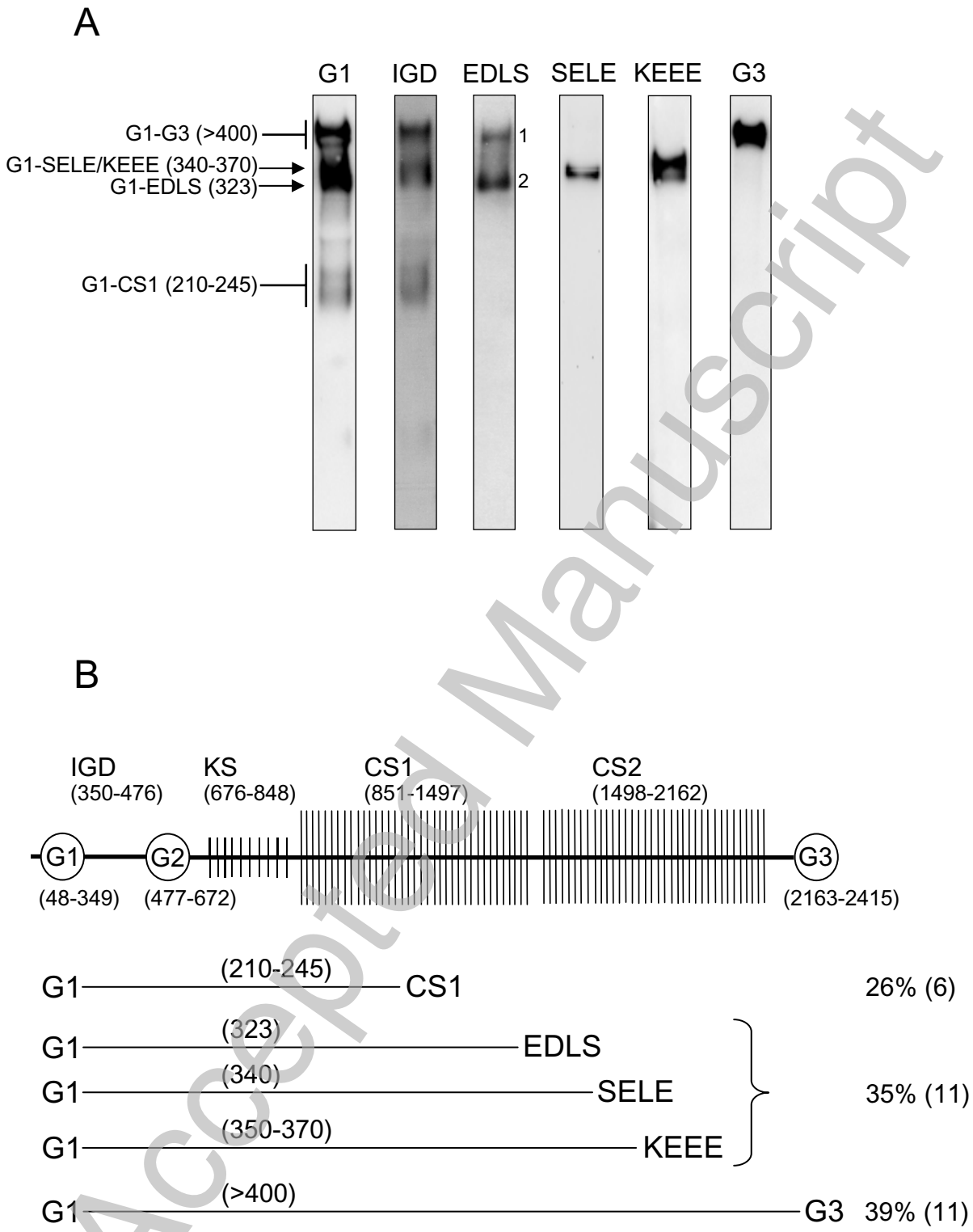

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Figure 2

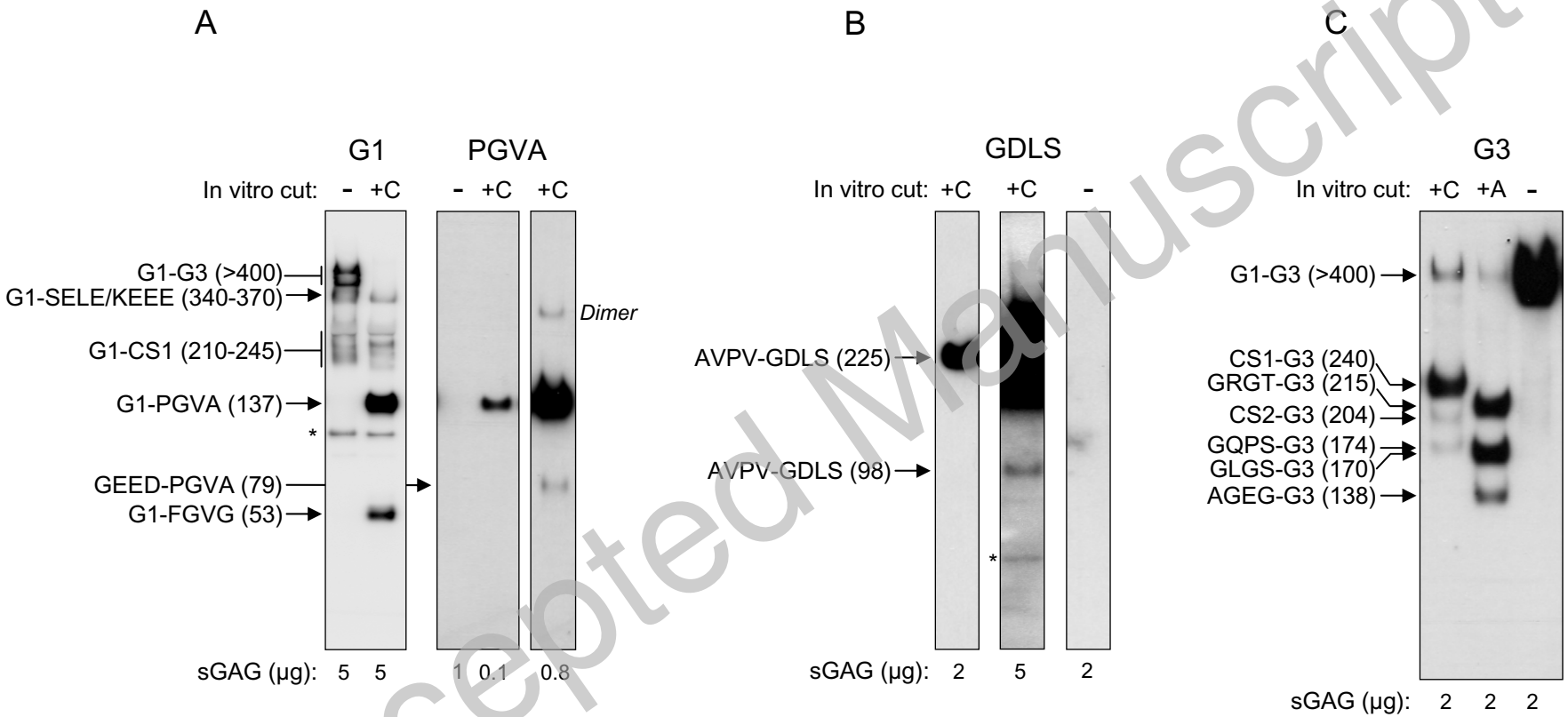

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Figure 3

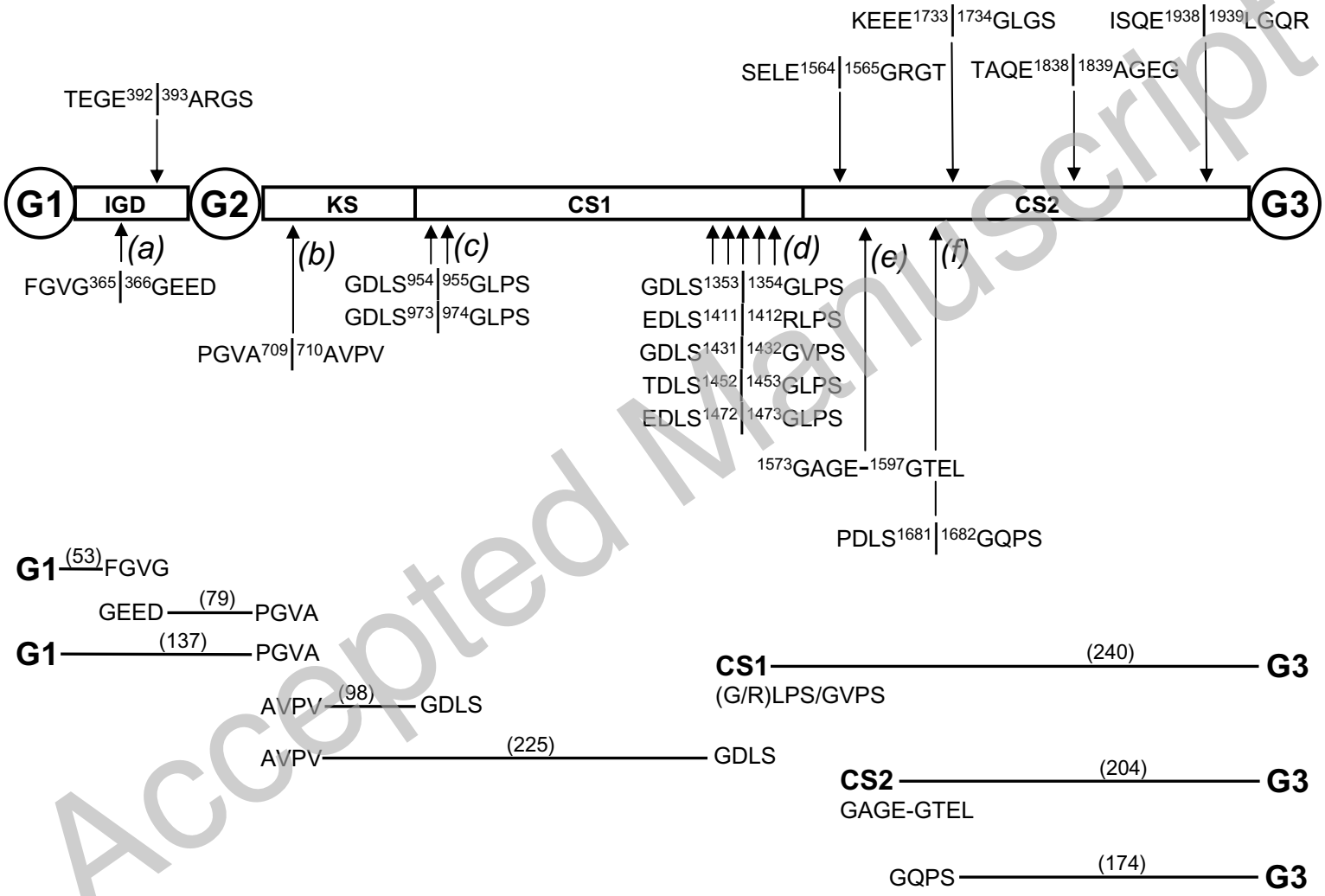

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Figure 4

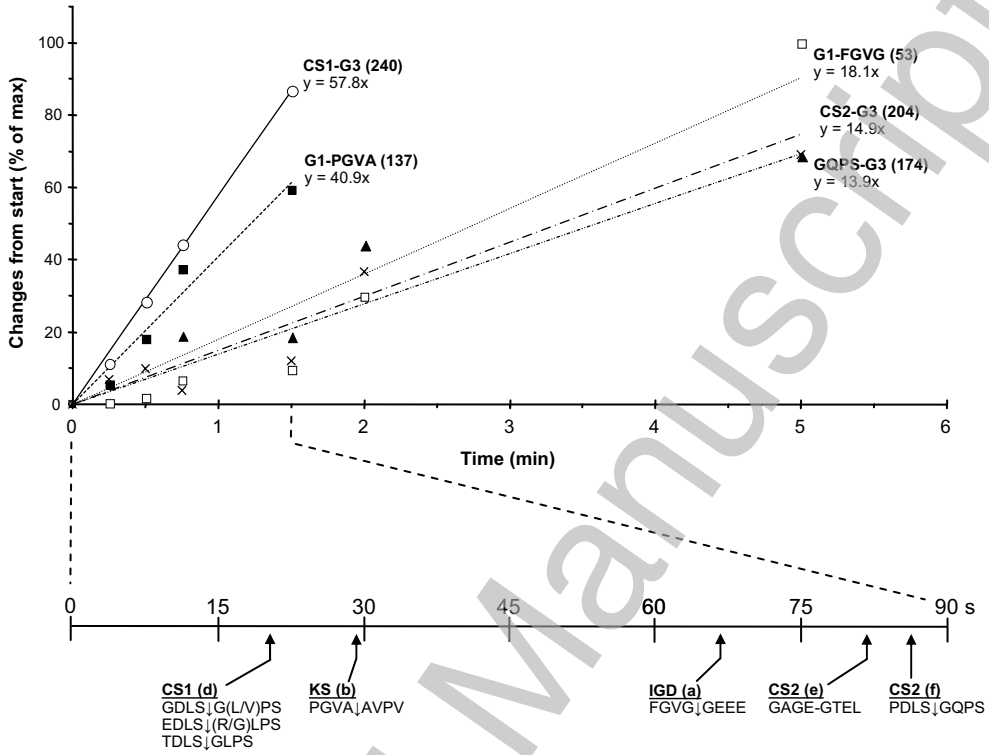

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Figure 5

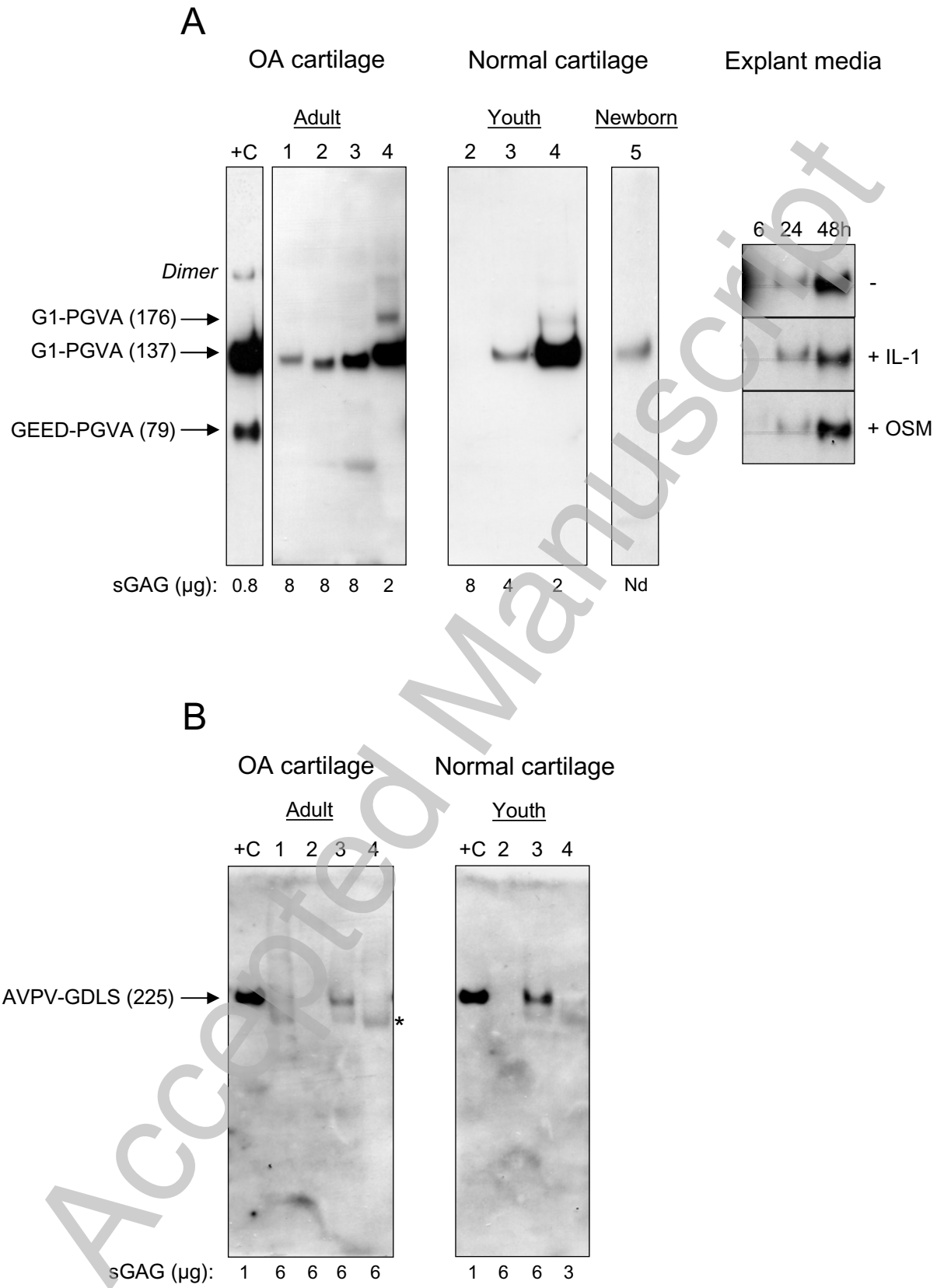

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Figure 5

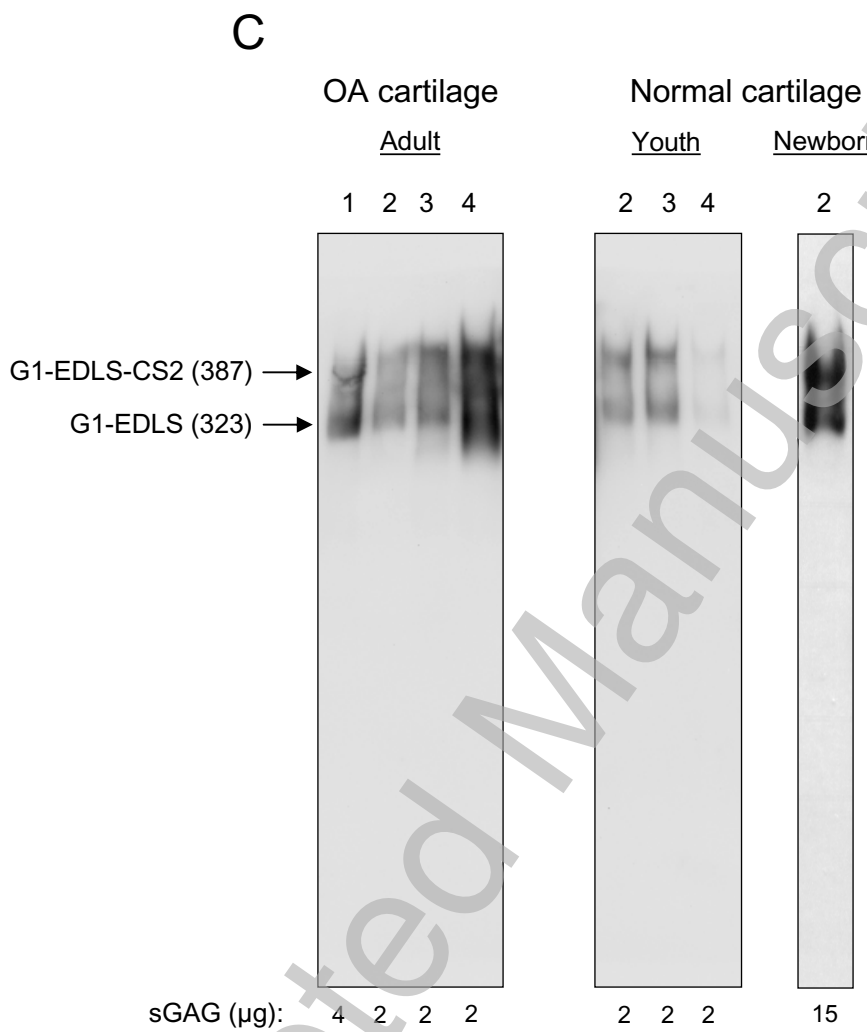

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Figure 6