Antibody-based exosite inhibitors of ADAMTS-5 (aggrecanase-2)

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Adamalysin-like metalloproteinases with thrombospondin (TS) motifs (ADAMTS)-5 is the multi-domain metalloproteinase that most potently degrades aggrecan proteoglycan in the cartilage and its activity is implicated in the development of osteoarthritis (OA). To generate specific exosite inhibitors for it, we screened a phage display antibody library in the presence of the zinc-chelating active site-directed inhibitor GM6001 (Ilomastat) and isolated four highly selective inhibitory antibodies. Two antibodies were mapped to react with exosites in the catalytic/disintegrin domains (Cat/Dis) of the enzyme, one in the TS domain and one in the spacer domain (Sp). The antibody reacting with the Sp blocked the enzyme action only when aggrecan or the *Escherichia coli*-expressed aggrecan core protein were substrates, but not against a peptide substrate. The study with this antibody revealed the importance of the Sp for effective aggrecanolytic activity of ADAMTS-5 and that this domain does not interact with sulfated glycosaminoglycans (GAGs) but with the protein moiety of the proteoglycan. An antibody directed against the Cat/Dis of ADAMTS-5 was effective in a cell-based model of aggrecan degradation; however, the anti-Sp antibody was ineffective. Western blot analysis of endogenous ADAMTS-5 expressed by human chondrocytes showed the presence largely of truncated forms of ADAMTS-5, thus explaining the lack of efficacy of the anti-Sp antibody. The possibility of ADAMTS-5 truncation must then be taken into account when considering developing anti-ancillary domain antibodies for therapeutic purposes.

Key words: adamalysin-like metalloproteinases with thrombospondin motifs (ADAMTS)-5, aggrecan, exosite inhibition, osteoarthritis, phage display, substrate selectivity.

INTRODUCTION

Adamalysin-like metalloproteinases with thrombospondin (TS) motif (ADAMTS)-5 is a multi-domain metalloproteinase comprising a large pro-domain, a catalytic domain (Cat), a disintegrin domain (Dis), a first TS (TS-1) domain, a cysteine-rich domain (CysR), a spacer domain (Sp) and a C-terminal second TS domain (TS-2). It cleaves several proteoglycans such as aggrecan, versican and brevican [1]. Aggrecan is a major component of articular cartilage and provides this tissue with the ability to resist compressive loads by creating high osmolarity of the tissue through hydration of sulfated glycosaminoglycan (GAG) chains. Dysregulated aggrecanolysis by extracellular proteases is considered to be crucial in the development of osteoarthritis (OA), the most prevalent joint disorder [2]. *In vitro* several ADAMTSs were shown to have aggrecanolytic activity [1], but ADAMTS-5 is by far the most potent aggrecanase characterized so far [3]. It effectively cleaves aggrecan core protein at several sites including the E^{392}–A^{393} bond (Uniprot accession number: P13608) located in the stretch between the first and the second globular domains, called the interglobular domain (IGD). This cleavage releases a large portion of aggrecan from the cartilage matrix and it is considered crucial for the development of OA. Since the studies showing that *Adamts-5* null mice are protected from cartilage degradation in an OA and an inflammatory-induced arthritis model were published [4,5], efforts have been made to develop small molecule inhibitors targeting this enzyme.

Most metalloproteinase inhibitors have been designed along with a zinc-chelating group such as hydroxamate or carboxylate [6]. However, since many metalloendopeptidases belonging to the so-called 'metzincin' superfamily share a similar topology around the active site zinc [7], chelation of this metal ion may lead to poor selectivity of such inhibitors. For example, the hydroxamate zinc-chelating inhibitor GM6001 (Ilomastat), originally designed to inhibit matrix metalloproteinases (MMPs), also inhibits members of the ADAMs and the ADAMTSs [8] and even metallopeptidases lacking any amino acid sequence homology with MMPs such as neprilysin, leucine aminopeptidase and dipeptidylpeptidase III [9]. These cross-inhibitions are considered to be responsible for musculoskeletal syndrome, a side effect caused by broad-spectrum MMP inhibitors and involving arthralgia, myalgia, joint stiffness and tendonitis [6]. One way to circumvent cross-inhibition is to target distal exosites that are less conserved than active sites [10]. In this regard, it is notable that the removal of the Sp domain dramatically reduces the aggrecanolytic activity of ADAMTS-5 and further removal of the CysR essentially

Abbreviations: ADAMTS, adamalysin-like metalloproteinases with thrombospondin motifs; Cat, catalytic domain; CysR, cysteine-rich domain; Dis, disintegrin domain; Fc, fragment crystallizable; GAG, glycosaminoglycan; *gst*, glutathione S-transferase; IGD, interglobular domain; LRP-1, low-density lipoprotein receptor-related protein-1; MMP, matrix metalloproteinase; MT1, membrane type-1 matrix metalloproteinase; N-TIMP-3, N-terminal inhibitory domain of TIMP3; OA, osteoarthritis; QF, quenched-fluorescent; RAP, receptor-associated protein; RU, response units; scFv, single chain variable fragment; Sp, spacer domain; SPR, surface plasmon resonance; TIMP-3, tissue inhibitor of metalloproteinase-3; TS, thrombospondin.

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abolished the activity, but not the activity for the general protease substrate *S*-carboxymethylated transferrin [3]. This suggests that these two non-catalytic ancillary domains must contain distal sites (i.e. exosites) that are involved in binding to the natural substrates such as aggrecan and versican [3]. It was postulated that molecules or antibodies that bind to those ancillary domains may selectively inhibit the activity of aggrecanases such as ADAMTS-4 and ADAMTS-5 against aggrecan, but not other substrates [11].

In the present work, we employed the phage display antibody technology based on a naive human lymphocyte library to obtain selective antibody-based inhibitors of ADAMTS-5. A phage display selection was carried out in the presence of the small molecule inhibitor GM6001 that binds to the active site of the enzyme in order to enhance the chance to obtain exosite-directed inhibitors and four distinct inhibitory antibodies have been isolated and characterized. Epitope mapping using various domain-deletion forms of ADAMTS-5 and competition experiments with known metalloproteinase inhibitors such as GM6001, tissue inhibitor of metalloproteinase-3 (TIMP-3) and its N-terminal inhibitory domain (N-TIMP-3) revealed that these antibodies react with sites well away from the catalytic site of ADAMTS-5. The study using the antibody against the Sp domain showed a new aggrecan-binding function of the Sp domain and its processing by human chondrocytes.

EXPERIMENTAL

Proteins

Flag-tagged human ADAMTS-5 and -4 deletion forms (Figure 1A) were expressed in stably transfected HEK293 cell lines as previously described [3]. Active concentrations of all enzymes were determined by titration against known concentrations of N-TIMP-3 using quenched-fluorescent (QF) peptides. Human full-length TIMP-3 was expressed and purified as previously reported [12]. Bovine nasal aggrecan was prepared according to the method of Hascall and Sajdera [13]. The Cat of MMP-1, MMP-2 and MMP-3 and receptor-associated protein (RAP) were prepared as described previously [12,14]. MMP-2, -7, -9, -10, MMP-13, MT1-MMP (membrane type-1 matrix metalloproteinase) and ADAM-17 were from Calbiochem. Unless stated otherwise, all chemicals were purchased from Sigma–Aldrich.

QF peptide cleavage assay

Antibody and enzyme were incubated in TNC buffer (50 mM Tris/HCl, pH 7.5, 100 mM NaCl, 10 mM $CaCl_2$, 0.02 % NaN_3 and 0.05 % Brij-35) for 2 h before addition of the appropriate substrate. Fluorometric assays were conducted at 37 °C in a Gemini microplate spectrofluorometer (Molecular Devices). The activity of ADAMTS-5 was monitored using the QF peptide substrate *o*-aminobenzoyl-T-E-S-E∼S-R-G-A-I-Y-(*N*-3-[2,4-dinitrophenyl]-L-2,3-diamino-propionyl)-K-K-NH_2 [Abz-TESE↓SRGAIY-Dpa-KK, '↓' indicates the cleavage site] (20 μM) with an excitation wavelength of 300 nm and an emission wavelength of 430 nm (420 nm cut-off). The activity of ADAMTS-4 was monitored using the QF peptide substrate carboxyfluorescein-A-E↓L-N-G-R-P-I-S-I-A-K-*N*,*N*,*N*′,*N*′-tetramethyl-6-carboxyrhodamine [Fam-AE↓LQGRPISIAK-Tamra (custom synthesized by Bachem) at a final concentration of 0.5 μM with an excitation wavelength of 485 nm and an emission wavelength of 538 nm (495 nm cut-off) [15]. The activities of MMPs and ADAM-17 were measured using the QF peptide substrate (7-methoxycoumarin-4-yl) acetyl-K-P-L-G↓L-(*N*-3-[2,4-dinitrophenyl]-L-2,3-diaminopropionyl)-A-R-NH_2 (Mca-KPLG↓L-Dpa-AR; FS-6) at 2 μM final concentration with an excitation wavelength of 325 nm and an emission wavelength of 400 nm [16]. Final concentrations of active enzymes were: 0.5 nM ADAMTS-5, 2 nM ADAMTS-4, 1 nM ADAM-17, 1 nM MMP-1 Cat domain, 1 nM MMP-2, 5 nM MMP-3, 2 nM MMP-7, 10 nM MMP-9, 10 nM MMP-10, 0.162 nM MMP-13, 1 nM MT1-MMP. All domain deletion mutants of ADAMTS-5 were incubated with substrate for 18 h at 37 °C before end-point reading. For all the other enzymes, after addition of the appropriate fluorogenic substrate, the hydrolysis was monitored every 15 s for 1 h. IC_{50} values were determined using the formula $v_i/v_0 = 1/(1 + [I]/IC_{50})$, where v_i is the initial velocity of substrate cleavage in the presence of the inhibitor at concentration [I] and v_0 is the initial velocity in the absence of the inhibitor.

Recombinant IGD substrate cleavage assay

Recombinant IGD substrate cleavage assay was performed essentially as previously described [17]. ADAMTS5-2 (Figure 1A, 0.05 nM) was incubated alone or with anti-ADAMTS-5 antibodies (total volume 5 μl) before addition of an equal volume *gst*-IGD-*flag* substrate consisting of glutathione S-transferase (*gst*) conjugated with the IGD sequence of aggrecan core protein (Y^{330}–G^{459}) and the C-terminal *flag* sequence (final concentration 17 μM) at 37 °C for 30 min. The reactions were stopped by addition of 2× SDS/PAGE sample buffer containing 10 mM sodium acetate–EDTA. Following SDS/PAGE (10 % gel) and staining with Coomassie Brilliant Blue R-250, the amount of product was determined by densitometric quantification of the 35-kDa band using the GS-710 scanning densitometer (Bio-Rad Laboratories) and analysed using the 1D Phoretix Software (Nonlinear Dynamics).

Aggrecan digestion assay

Aggrecan digestion assay was performed as previously described [8]. Briefly, 50 μg of aggrecan (final concentration 670 nM) were incubated with ADAMTS5-2 (2 pM for cleavage at E^{1790}–A^{1791} site, 0.5 nM for cleavage at E^{392}–A^{393} site) in TNC buffer at 37 °C for 2 h. The reaction was stopped with EDTA buffer (200 mM sodium acetate, 250 mM Tris/HCl pH 8.0 and 100 mM EDTA). Aggrecan was incubated with 0.1 milliunits/μl of chondroitinase ABC and 0.1 milliunits/μl of keratanase (Seikagaku) overnight at 37 °C to remove GAG chains. The samples were precipitated with cold acetone, incubated at –20 °C for 4 h and then centrifuged at 13 000 ***g*** for 30 min. The dried pellet was dissolved in reducing sample buffer, run on SDS/PAGE (6 % gel) and analysed by Western blotting using Trans-Blot® TurboTM Transfer System (BioRad) according to the manufacturer's instructions. Membranes were probed with rabbit polyclonal anti-AGEG antibody (which detects aggrecanase cleavage at the E^{1790}–A^{1791} bond) [18] or mouse monoclonal BC-3 antibody (which detects aggrecanase cleavage at E^{392}–A^{393} bond, Abcam).

Chondrocyte monolayer assay for aggrecan degradation

Chondrocytes were isolated as described previously [18]. Human articular cartilage was obtained from patients undergoing amputations at the Royal National Orthopaedic Hospital (Stanmore, UK) following informed consent and approval by the Riverside Research Ethics Committee. Healthy cartilage was obtained from the knee after amputation due to soft tissue

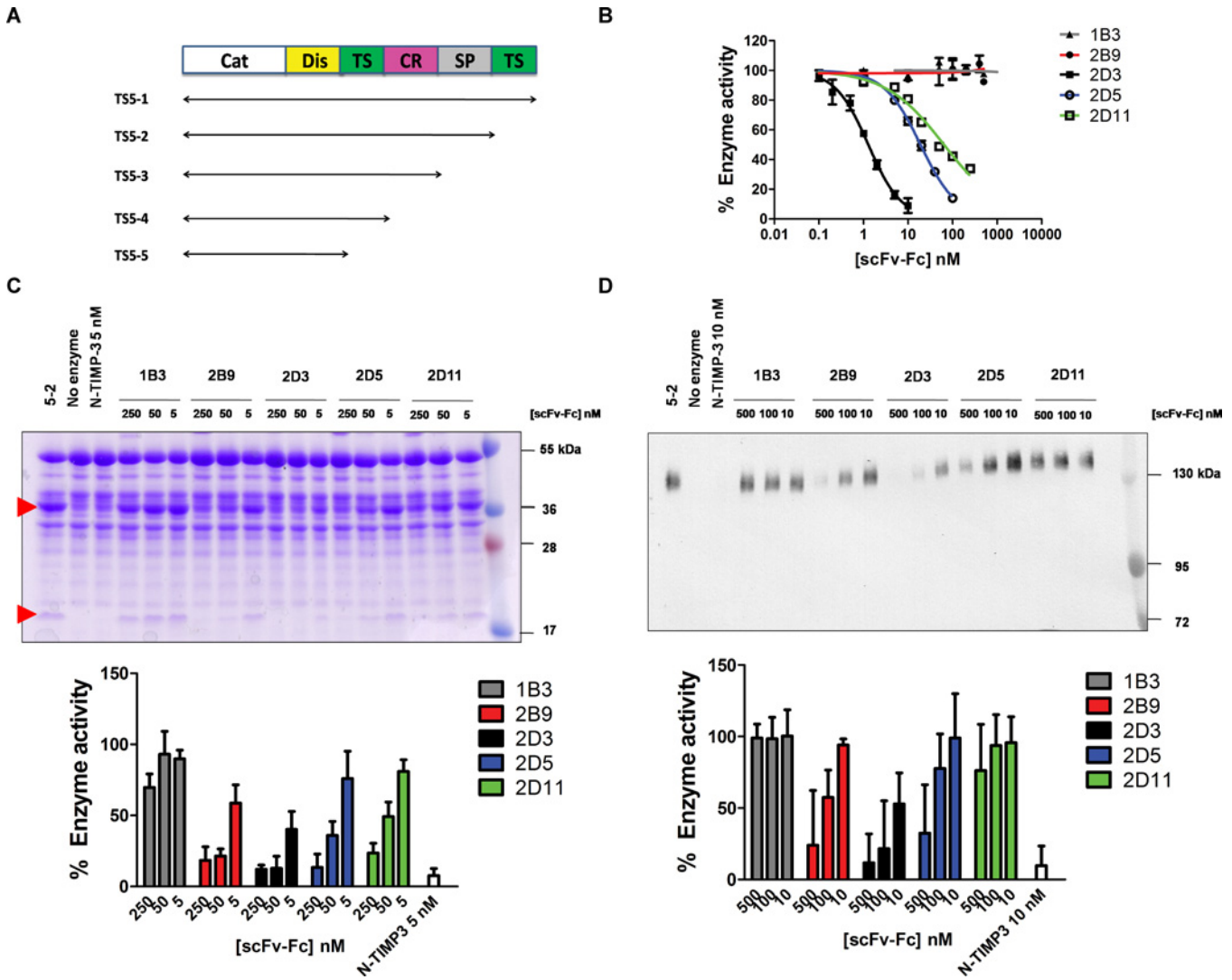


Figure 1 Inhibition of ADAMTS-5 by scFv–Fc antibodies

(**A**) Schematic representation of domain deletion mutants of ADAMTS-5. CR, CysR. (**B**) Inhibition of QF peptide cleavage. The curves are the mean ± S.E.M (n=5). (**C**) Inhibition study using recombinant *gst*-IGD-*flag* substrate. In the presence of ADAMTS-5, the full-length substrate was converted into a *gst*-NITEGE fragment (~35 kDa) and an ARGSV-*flag* fragment (~17 kDa) as a result of cleavage at E^{392}–A^{393} bond. The 35-kDa fragment was quantified by densitometric analysis (n=3). (**D**) Inhibition study using bovine aggrecan. Fragments cleaved at E^{1790}–A^{1791} bond were detected by a polyclonal neoepitope antibody recognizing the new N-terminus fragment (n=3).

sarcoma and osteosarcoma with no involvement of the cartilage. Cells were plated at a density of 2.5×10^5 cells/well (24-well plate) in Dulbecco's modified Eagle Medium supplemented with 10 % FBS. To evaluate aggrecanolysis, chondrocytes were rested for 24 h in serum-free medium then cultured for 24 h in the presence or absence of antibodies, N-TIMP-3 and bovine aggrecan (100 μg/ml). To evaluate aggrecan degradation products in chondrocytes cultures, 200 μl of conditioned medium were collected for Western blot analysis. For detection of endogenous ADAMTS-5, chondrocytes were incubated for 48 h in the presence and absence of RAP (500 nM). Media were collected, supplemented with protease inhibitor cocktail and metzincin inhibitor CT-1746 (CellTech), concentrated with a Vivaspin device (Sartorius, cut-off 3 kDa) and run under non-reducing conditions in a 4 %–12 % BisTris gel (Life Technologies). Viability was tested by MTS assay (Promega). All data were analysed by unpaired one-tail t tests with Welch's correction using the software package GraphPad Prism.

Phage display selections

Human recombinant ADAMTS5-2 was biotinylated with EZ-link Sulpho-N-Hydroxyssuccinimide-LC biotin (Pierce Biotechnology); the degree of labelling was estimated to be 1.5 (on average 1.5 molecules of biotin/molecule of enzyme) (Fluoreporter Biotin Quantitation Assay Kit, Invitrogen). The biotinylated enzyme was tested for wild-type aggrecanase and peptidolytic activity and exposed (100 nM) to a naive human single chain variable fragment (scFv) phage-display library [19] in the presence of GM6001 (Ilomastat, N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide, Elastin Product Co; 10 μM). The library was previously deselected against *flag* peptide (5 μM) to remove phages recognizing the *flag*-tag on the recombinant antigen. Following two rounds of solution-phase selection, the eluted polyclonal scFv population was cloned into pBIOCAM5 expression vector for mammalian expression of scFv–Fc fusions (scFv fused with the fragment

crystallizable region of immunoglobulin) and transformed into DH5α *Escherichia coli* [20]. Individual clones were isolated and used as a source of DNA for transient transfection in HEK293-F cells (Invitrogen) [20]. Conditioned medium was then screened by ELISA against biotinylated recombinant ADAMTS5-2 (50 nM) bound to streptavidin-coated Black Nunc MaxiSorp™ plates in the absence of GM6001. Bound antibodies were detected by time-resolved fluorescence using europium-labelled anti-human antibody (PerkinElmer) and dissociation-enhanced lanthanide fluorescence immunoassay enhancement solution (PerkinElmer) followed by europium signal acquisition (330 nm excitation, 620 nm emission) on a plate reader.

Following initial screening, selected anti-ADAMTS-5 clones were expressed in 25-ml scale and purified by immobilized metal affinity chromatography [20].

Solid-phase-binding assay for ADAMTS-5 binding to heparin

Heparin (10 μg/ml TNC) was coated overnight at 25 °C on to heparin-binding plates (Beckton Dickinson). Wells were washed in TNC buffer containing 0.1 % Tween-20 between each subsequent incubation. Wells were blocked with 0.2 % gelatin in TNC buffer and then incubated with recombinant biotinylated ADAMTS-5 in blocking solution for 2 h at 37 °C. Bound proteins were detected using streptavidin coupled to horseradish peroxidase for 1 h at 37 °C. Hydrolysis of tetramethylbenzidine substrate was measured at 450 nm.

Surface plasmon resonance

Surface plasmon resonance (SPR) measurements were carried out on a BIAcore-T100 instrument using anti-human IgG capture kit or covalent immobilization on CM5 biosensor chips (GE Healthcare). Rabbit anti-human IgG was chemically immobilized on CM5 biosensor chips by amine coupling and the anti-ADAMTS-5 antibodies were captured to give ∼300 response units (RU). ADAMTS-5 (0.2–100 nM) was injected in TNC buffer containing 0.05 % Tween-20 at 25 °C with a flow rate of 30 μl/min. For competition studies, TIMP-3 (50 nM), N-TIMP-3 (50 nM) or GM6001 (100 μM) was pre-incubated for 1 h with ADAMTS-5 before injection. The surface of the chip was regenerated after each injection of sample with 3 M $MgCl_2$ (40 s, 30 μl/min). Capture level of each antibody did not vary significantly between running cycles, indicating that the performance of the chip remained stable. Furthermore, at least one non-zero concentration of ADAMTS-5 was run twice for each kinetic experiment. This yielded identical binding curves, confirming that the setup was stable. For competition studies between anti-ADAMTS-5 antibodies, 2B9 was amine-coupled to a CM5 chip (∼300 RU) and titrated concentrations of ADAMTS-5 in the presence of 200 nM of competitor antibody were injected. The surface of the chip was regenerated with 10 mM glycine, pH 2.5. Binding curves were corrected for background binding to a non-coated control channel. Association rate constants (k_{on}) and dissociation rate constants (k_{off}) were obtained by using a simple one-to-one binding model (BIACore Evaluation Software). Chi-square values were $\leqslant 10$.

Modelling

A simple model of ADAMTS-5–TIMP-3 was constructed based on available structures using Pymol. The Cat/Dis structure of ADAMTS-5 (PDB entry: 2RJQ [21]) was used as a starting point. The ADAM-17–N-TIMP-3 complex (PDB entry: 3CKI [22]) was superimposed on the metalloproteinase signature sequence (HEXXHXXGXXH . . . M) to derive a likely position for N-TIMP-3. Subsequently, the MMP-3–TIMP-1 complex (PDB entry: 1UEA [23]) was superimposed and the C-terminal domain of TIMP-1 used to define the likely position of the TIMP-3 C-terminal domain. Finally, in the absence of crystallographic data for ADAMTS-5 ancillary domains, the crystal structure of ADAMTS-13 (3GHN) [24] containing the TS-1, CysR and Sp domains was superimposed on the ADAMTS-5 structure to give an idea of fold and orientation of these domains relative to the Cat/Dis domains.

RESULTS

Screening for inhibitory antibodies of ADAMTS-5

Recombinant human ADAMTS-5 devoid of the C-terminal TS domain (ADAMTS5-2; Figure 1A) was purified, biotinylated and exposed to a naive human scFv antibody phage display library with a diversity of 10^{10} clones [19] in the presence of the low molecular mass general MMP inhibitor GM6001 (10 μM). An excess of this inhibitor was used to negatively-select scFv antibodies with epitopes localized in the proximity of the active-site zinc and in the S1′-substrate specificity pocket (nomenclature after Schechter and Berger [25]). After two rounds of solution-phase selection, the polyclonal antibody population was subcloned into a mammalian vector for liquid-phase expression in HEK293F cells where antibodies were secreted as fusion proteins with the Fc(fragment crystallizable) region of human IgG1 (scFv-C_H2-C_H3 dimers or scFv–Fc) [20]. Three-hundred clones were screened by ELISA. Among them, 116 clones were high-binders and, on the basis of sequence difference, 51 clones were chosen to be expressed in mammalian cells, purified and tested for their ability to inhibit ADAMTS5-2 activity against a QF peptide substrate and bovine aggrecan. This screening discriminated between antibodies that bind to Cat/Dis domains of ADAMTS5-2 (i.e. inhibitors of QF peptide cleavage) and those that bind to distal ancillary domains and inhibit aggrecanolytic activity only. Among the tested antibodies, 2D3, 2D5 and 2D11 (Supplementary Table S1 for the amino acid sequence) showed high inhibitory activity against ADAMTS-5 using the QF substrate with IC_{50} values in low nanomolar ranges (Figure 1B; Table 1). These antibodies also inhibited cleavage of an *E. coli*-expressed unglycosylated recombinant *gst*-IGD-*flag* containing the human aggrecan sequence Y^{349}–G^{476} with IC_{50} values similar to those observed with the QF substrate (Figure 1C; Table 1). Both 2D3 and 2D5 were able to inhibit aggrecan cleavage as demonstrated by neo-epitope antibodies detecting cleavage at the E^{392}–A^{393} and E^{1790}–A^{1791} sites, but their IC_{50} values were significantly higher than those on the QF substrate (Table 1). 2D11 was not effective in inhibiting aggrecan cleavage (Table 1). Antibody 2B9 did not inhibit the activity against the QF peptide substrate (Figure 1B), but it effectively inhibited the cleavage of both *gst*-IGD-*flag* and native aggrecan (IC_{50} values of 8 and 140 nM for the E^{392}–A^{393} site respectively; Figure 1C; Table 1). Antibody 1B3 that bound to ADAMTS-5 with $K_D = 100$ nM was used as a negative control, as it did not inhibit ADAMTS-5 activity against any substrate (Figures 1B–1D).

Antibodies 2D3, 2D5 and 2D11 did not inhibit ADAMTS-4, nor a panel of metalloproteinases (MMP-1,-2, -3, -7, -9, -10, -13, MT1-MMP and ADAM-17), in the peptide cleavage assay (result not shown). 2B9 did not inhibit the aggrecanase activity of ADAMTS-4 (Supplementary Figure S1). When their specificity was tested by SPR, none of the four antibodies bound either to ADAMTS-4 or to ADAM-17 (result not shown).

Table 1 IC_{50} values of lead antibodies against various domain-deletion mutants of ADAMTS-5

The inhibitory activity of anti-ADAMTS-5 antibodies was measured against QF peptide (Abz-TESE∼SRGAIY-Dpa-KK), *E. coli*-expressed recombinant *gst*-IGD-*flag* substrate (IGD) and bovine aggrecan as described in 'Experimental'. Ni, not inhibiting at 500 nM. Data are IC_{50} values (nM) of the mean data ± S.E.M. (n=3–5).

	QF peptide				IGD	Aggrecan E^{392}–A^{393}	Aggrecan E^{1790}–A^{1791}
Clone	TS5-2	TS5-3	TS5-4	TS5-5	TS5-2	TS5-2	TS5-2
2B9	Ni	Ni	Ni	Ni	8.2 ± 2.8	140 ± 50	90 ± 10
2D3	2.5 ± 0.6	3.2 ± 0.2	5.5 ± 0.3	8.3 ± 0.8	2.1 ± 0.9	90.0 ± 50	8.0 ± 1.0
2D5	20 ± 5	37 ± 2.5	35 ± 2.6	53 ± 2.8	23 ± 0.8	400 ± 200	200 ± 100
2D11	70 ± 8	79 ± 16	73 ± 11	130 ± 31	46 ± 2	Ni	Ni

Mapping of the domains that react with anti-ADAMTS-5 antibodies

To identify the domain(s) of ADAMTS-5 that interact with each antibody, a series of domain deletion mutants of ADAMTS-5 (Figure 1A) were expressed and purified [3]. Since the smallest enzyme with detectable proteolytic activity consists of the Cat/Dis domains [3] and the crystallographic data indicate that these two domains are structurally integrated [21], we used the Cat/Dis domains (ADAMTS5-5) as a minimal protease. When tested in the QF peptide cleavage assay, antibodies 2D3, 2D5 and 2D11 showed small increases in IC_{50} value against domain-deleted variants (Table 1). These values were in good agreement with their dissociation constant (K_D) values determined by SPR (Table 2). 2B9 bound to ADAMTS5-2 with relatively high affinity (K_D value of 6.6 nM), but it did not bind to any of the other domain-deletion forms of ADAMTS-5, even at the highest concentration tested (200 nM), indicating that the Sp domain is the main epitope for 2B9 (Table 2; Supplementary Figure S2). This was further confirmed in that 2B9 did not inhibit ADAMTS5-3 activity against bovine aggrecan or *gst*-IGD-*flag* (result not shown). When 2B9 was immobilized on the surface of a biosensor chip, it bound to ADAMTS5-2 with a K_D value of 9.7 nM and this binding was not significantly affected by the presence of 200 nM of 2D3, 2D5 or 2D11, suggesting that the two types of antibodies bind independently (Supplementary Table S2). 2D3, 2D5 and 2D11 bound to both ADAMTS5-2 and ADAMTS5-5, but there were some differences in the mode of interaction. 2D3 and 2D11 bound with essentially the same affinity to all domain-deletion mutants tested, indicating that their epitopes are in the Cat/Dis domain. On the other hand, antibody 2D5 exhibited a 3-fold increase in K_D for ADAMTS5-5 compared with ADAMTS5-4 (Table 2; Supplementary Figure S2), which mirrored the inhibition potency of 2D5 (Table 1), indicating the importance of the TS-1 domain for 2D5 binding. To further investigate the epitope of 2D5, domain-deletion mutants of ADAMTS-5 were subjected to Western blot analysis under non-reducing conditions. All forms containing the TS-1 domain reacted with 2D5, but not ADAMTS5-5 (Figure 2). Taking into account the differences between the SPR and the Western blot data (i.e. native epitope compared with linear epitope respectively) we concluded that the epitope lies at the interface between the Cat/Dis and the TS-1 domain.

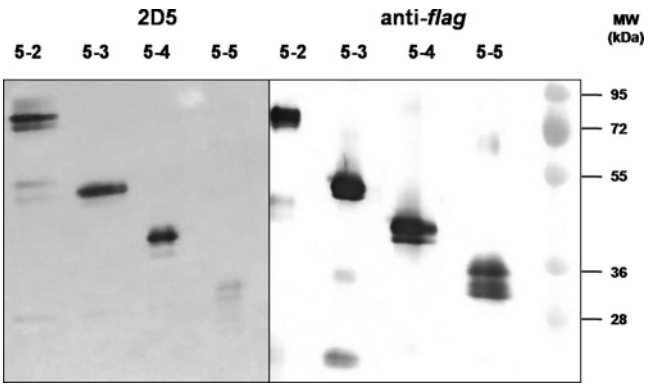


Figure 2 Western blot of domain-deletion forms of ADAMTS-5 with 2D5

Various forms of ADAMTS-5 (115 ng) were run under non-reducing conditions on a 10% acrylamide gel and 2D5 (100 nM) or anti-*flag* were used as primary antibodies. Secondary alkaline phosphatase conjugated antibodies were anti-human (for 2D5) or anti-mouse (for anti-*flag*).

Effect of TIMP-3 on the interactions between ADAMTS-5 and antibodies

The presence of GM6001, the zinc-chelating small molecule inhibitor used during the phage display selection, did not affect the binding of anti-ADAMTS-5 antibodies to ADAMTS-5 (Table 3). To further investigate the epitopes of the antibodies, we used TIMP-3 in SPR analysis (Table 3; Supplementary Figure S3). 2B9, 2D5 and 2D11 showed small changes of their binding affinity to ADAMTS5-2 in the presence of TIMP-3, but a large decrease (∼40-fold) in affinity was observed with 2D3, suggesting that TIMP-3 and 2D3 have overlapping binding sites. To further map the 2D3 epitope, we used the N-terminal domain of TIMP-3 which lacks the C-terminal domain of 68 amino acids (N-TIMP-3). N-TIMP-3 forms tight complexes with MMPs, ADAMs and ADAMTSs [26]. SPR analysis showed ∼2–3-fold increase in binding affinity for 2B9 and 2D11 and ∼6-fold increase for 2D5 in the presence of N-TIMP-3, suggesting that conformational changes may occur around their binding site upon binding of ADAMTS-5 to N-TIMP-3 (Table 3; Supplementary Figure S3). However, the binding affinity of 2D3 was not affected by N-TIMP-3 binding (Table 3; Supplementary Figure S3). The modelling of the complex formed between ADAMTS5-2 and TIMP-3 showed that the C-terminal domain of TIMP-3 could interact with the Dis domain of the enzyme (Supplementary Figure S4), suggesting that the epitope of 2D3 is primarily located in the Dis domain.

Interactions between ADAMTS-5 Sp domain and aggrecan are not affected by the presence of GAGs

The most unexpected result was that 2B9 inhibited the activity of ADAMTS5-2 on *E. coli*-expressed unglycosylated *gst*-IGD-*flag* (Figure 1C), because the C-terminal Sp and CysR domains of ADAMTS-4 and -5 are essential for effective aggrecanolytic activity and we previously reported that the Sp domain of

Table 2 Analysis of ADAMTS-5 binding to scFv–Fc antibodies by SPR

Anti-ADAMTS-5 antibodies were captured by an anti-human antibody on the surface of a CM5 chip and different concentrations of antigen (0.2–200 nM) were injected. Association rate constant (k_{on}) and dissociation rate constant (k_{off}) and the equilibrium dissociation constant (K_D) were determined by global fitting of the SPR data to a 1:1 binding model. NB, no measurable binding at 200 nM antigen. Mean ± S.E.M. are reported (n=3). Subscript 'x' in $K_{D\,5\text{-}x}$ indicates the K_D value of a specified ADAMTS-5 deletion mutant where x is –3, –4 or –5.

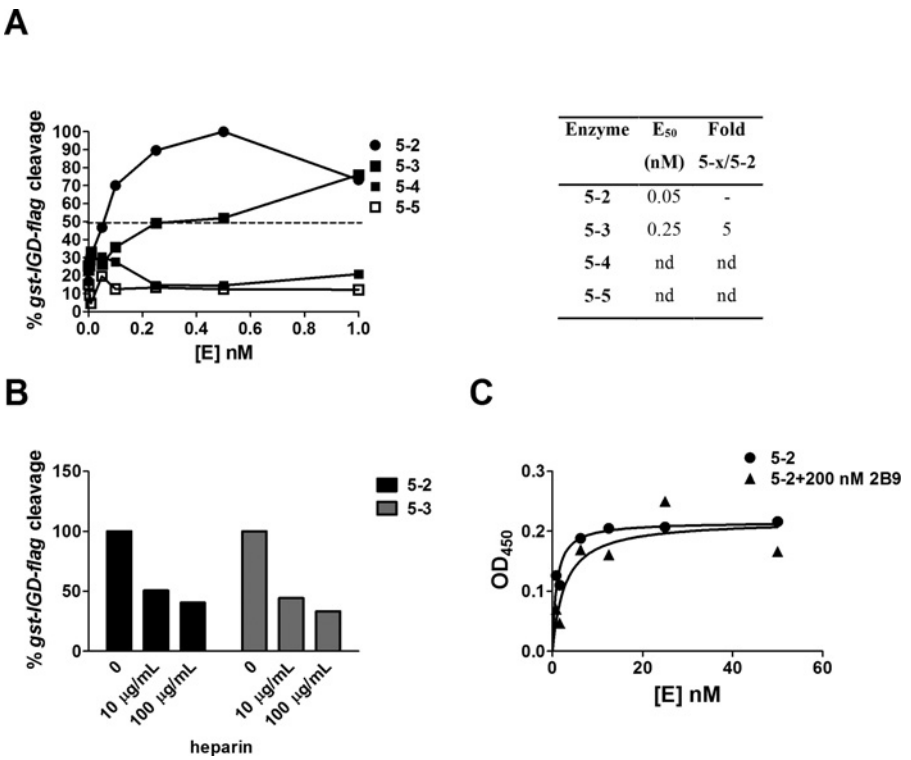
	k_{on} (10^5 $M^{-1} \cdot s^{-1}$)	k_{off} (10^{-3} s^{-1})	K_D (nM)	Ratio $K_{D\,5\text{-}2}/K_{D\,5\text{-}x}$	Epitope
2B9					Sp
ADAMTS5-2	25 ± 3	16 ± 2	6.6 ± 0.7	1.0	
ADAMTS5-3	–	–	NB	–	
ADAMTS5-4	–	–	NB	–	
ADAMTS5-5	–	–	NB	–	
2D3					Dis/Cat
ADAMTS5-2	2.6 ± 0.9	0.7 ± 0.1	3.9 ± 1.2	1.0	
ADAMTS5-3	2.5 ± 0.6	1.3 ± 0.3	5.3 ± 0.1	0.74	
ADAMTS5-4	2.6 ± 0.7	1.3 ± 0.3	5.0 ± 0.1	0.78	
ADAMTS5-5	3.9 ± 1.2	1.0 ± 0.1	3.9 ± 1.2	1.0	
2D5					TS-1/Dis/Cat
ADAMTS5-2	1.9 ± 0.7	5.8 ± 0.5	32 ± 11	1.0	
ADAMTS5-3	3.3 ± 0.1	6.7 ± 0.2	21 ± 2.0	1.5	
ADAMTS5-4	6.9 ± 3.1	17 ± 4	25 ± 5.8	1.3	
ADAMTS5-5	2.1 ± 1.1	14 ± 4	76 ± 26	0.4	
2D11					Dis/Cat
ADAMTS5-2	15 ± 10	38 ± 7	34 ± 18	1.0	
ADAMTS5-3	21 ± 0.1	69 ± 10	34 ± 3.7	1.0	
ADAMTS5-4	35 ± 7	120 ± 20	33 ± 2.2	1.0	
ADAMTS5-5	35 ± 25	86 ± 50	27 ± 4.0	1.3	

Table 3 Effect of GM6001, TIMP-3 and N-TIMP-3 on binding of scFv–Fc antibodies to ADAMTS5-2 using SPR analysis

Association and dissociation rate constants (k_{on} and k_{off} respectively) and the equilibrium dissociation constant (K_D) were determined by global fitting of the SPR data to a 1:1 binding model. Mean ± S.E.M. are reported (n=3).

	k_{on} (10^5 $M^{-1} \cdot s^{-1}$)	k_{off} (10^{-3} s^{-1})	K_D (nM)	Ratio $K_D{}^+/K_D{}^-$
2B9				
Free ADAMTS5-2	29 ± 1	20 ± 2	6.9 ± 0.8	
+100 μM GM6001	26 ± 1	15 ± 0.5	5.6 ± 0.5	0.81
+50 nM TIMP-3	13 ± 1	5.2 ± 0.1	4.2 ± 0.4	0.61
+50 nM N-TIMP-3	32 ± 1	7.4 ± 0.1	2.3± 0.4	0.33
2D3				
Free ADAMTS5-2	1.6 ± 0.5	0.7 ± 0.07	4.9 ± 1.0	
+100 μM GM6001	1.0 ± 0.1	0.8 ± 0.01	7.8 ± 1	1.6
+50 nM TIMP-3	0.06 ± 0.006	0.9 ± 0.01	154 ± 2	37
+50 nM N-TIMP-3	0.8 ± 0.06	0.6 ± 0.03	7.6 ± 0.5	1.6
2D5				
Free ADAMTS5-2	1.4 ± 0.2	5.6 ± 0.9	41 ± 0.9	
+100 μM GM6001	1.9 ± 0.03	6.6 ± 0.1	35 ± 3	0.85
+50 nM TIMP-3	1.4 ± 0.01	10 ± 3	76 ± 19	1.9
+50 nM N-TIMP-3	2.8 ± 0.01	2.1 ± 0.5	7.2 ± 2	0.18
2D11				
Free ADAMTS5-2	5.9 ± 0.1	35 ± 0.3	60 ± 0.4	
+100 μM GM6001	9.7 ± 0.2	40 ± 0.6	41 ± 3	0.68
+50 nM TIMP-3	11 ± 0.5	40 ± 2	38 ± 4	0.63
+50 nM N-TIMP-3	9.9 ± 0.1	29 ± 0.3	29 ± 3	0.48

ADAMTS-4 [27,28] and the CysR domain of ADAMTS-5 [3] interact with sulfated GAGs of aggrecan. Since the exact role of the Sp domain of ADAMTS-5 in the cleavage of recombinant *gst*-IGD-*flag* is not known, we compared activities of ADAMTS5-2 and ADAMTS5-3 on this substrate. As shown in Figure 3A, ADAMTS5-2 was more active than ADAMTS5-3 on *gst-IGD-flag*, although there was a decrease in ADAMTS5-2 activity at higher concentrations above 0.25 nM, which is due to the lack of linearity of the assay at above 75 % digestion of the substrate. We therefore took the enzyme amount that digests 50 % of the substrate (E_{50}) to compare the enzyme activities. The results showed that ADAMTS5-2 is approximately five times more active than ADAMTS5-3, indicating that the Sp domain plays an important role in ADAMTS-5 cleavage of the E^{392}–A^{393} bond in the IGD and that its interaction with the IGD is not governed by GAGs. Deletion of the CysR domain essentially abolished the activity on *gst*-IGD-*flag*. Activities of both ADAMTS5-2 and ADAMTS5-3 on *gst*-IGD-*flag* were



Enzyme	E_{50} (nM)	Fold 5-x/5-2
5-2	0.05	-
5-3	0.25	5
5-4	nd	nd
5-5	nd	nd

Figure 3 The Sp domain of ADAMTS-5 mediates protein–protein interactions with recombinant *gst-IGD-flag* substrate

(**A**) Cleavage of recombinant *gst*-IGD-*flag* substrate by ADAMTS-5 deletion forms. Graph shows densitometric analysis of NITEGE bands. To estimate the enzymatic activity, the enzyme concentration that gives 50 % of the total substrate digestion (E_{50}) was estimated. Results are shown as the mean of duplicates. nd, not determined. (**B**) Inhibition of IGD cleavage by heparin. ADAMTS5-2 (0.05 nM) and 5-3 (0.5 nM) were incubated with heparin for 2 h before addition of the IGD-*flag* substrate. (**C**) 2B9 does not significantly affect binding of ADAMTS5-2 to heparin. Biotinylated ADAMTS5-2 (0–50 nM) was incubated with heparin-coated wells in the presence and absence of 200 nM 2B9 and the binding of ADAMTS5-2 was detected by streptavidin-conjugated horseradish peroxidase activity. Values are corrected for binding of biotinylated ADAMTS5-2 to non-coated wells. OD, absorbance.

inhibited by heparin to a similar extent (Figure 3B), but the binding of ADAMTS5-2 to heparin was not blocked by 2B9 (Figure 3C), indicating that heparin binds to the CysR domain, but not the Sp domain.

Effect of anti-ADAMTS-5 antibodies on aggrecan degradation by human chondrocytes in culture

We then examined the effect of anti-ADAMTS-5 antibodies on aggrecan degradation using a cell-based assay, where human chondrocytes were cultured for 24 h in the presence of exogenously added bovine aggrecan as substrate. We chose 2D3 and 2B9 as these antibodies represent two different modes of inhibitory action on aggrecan degradation. No effect on cell viability was observed up to 100 nM (Supplementary Figure S5). As shown in Figure 4(A), addition of N-TIMP-3, an inhibitor of ADAMTS-4 and ADAMTS-5, inhibited completely aggrecan degradation at 10 nM. 2D3 partially inhibited aggrecan degradation at 10 nM and ∼80 % inhibition was observed at 100 nM. However, 2B9 was not effective at 100 nM (Figure 4A) and even at 500 nM (result not shown). To investigate the reason for this lack of efficacy, we analysed the chondrocyte culture medium to assess the molecular forms of endogenous ADAMTS-5.

The detection of extracellular ADAMTS-5, even in culture conditions, is challenging, as the newly synthesized secreted enzyme is rapidly endocytosed by the scavenger receptor, low-density lipoprotein receptor-related protein-1 (LRP1) [29]. To prevent LRP1-mediated internalization, RAP (500 nM), a ligand-binding antagonist of LRPs, was added to chondrocyte cultures for 48 h and the medium was collected, supplemented with protease inhibitors and analysed by Western blot using an in-house monoclonal antibody against the Cat/Dis domain of ADAMTS-5, 2A11 (Figure 4B). The prevalent form of ADAMTS-5 in the culture medium lacked the Sp domain (Figure 4B). In the absence of RAP, no ADAMTS-5 was detected in the culture medium.

DISCUSSION

ADAMTS-5 is a multi-domain metalloproteinase and its ancillary domains are essential for aggrecanolytic activity [3]; therefore, both the Cat and the ancillary domains are potential targets for inhibitory antibody development. In the present study, we have isolated four different types of antibodies for ADAMTS-5 and characterized them in terms of their interaction sites and inhibitory activities, which is summarized in Figure 5. Two of the four antibodies, 2D3 and 2D11, react with epitopes in the Cat/Dis domains, but neither are directed to the active site. 2D3 is the strongest inhibitor among the four when QF peptide and recombinant *gst*-IGD-*flag* are used as substrates, but the IC_{50} value against aggrecan was significantly higher. This is mainly due to the different affinity of ADAMTS-5 for these substrates (see below). The binding competition studies with TIMP-3 and

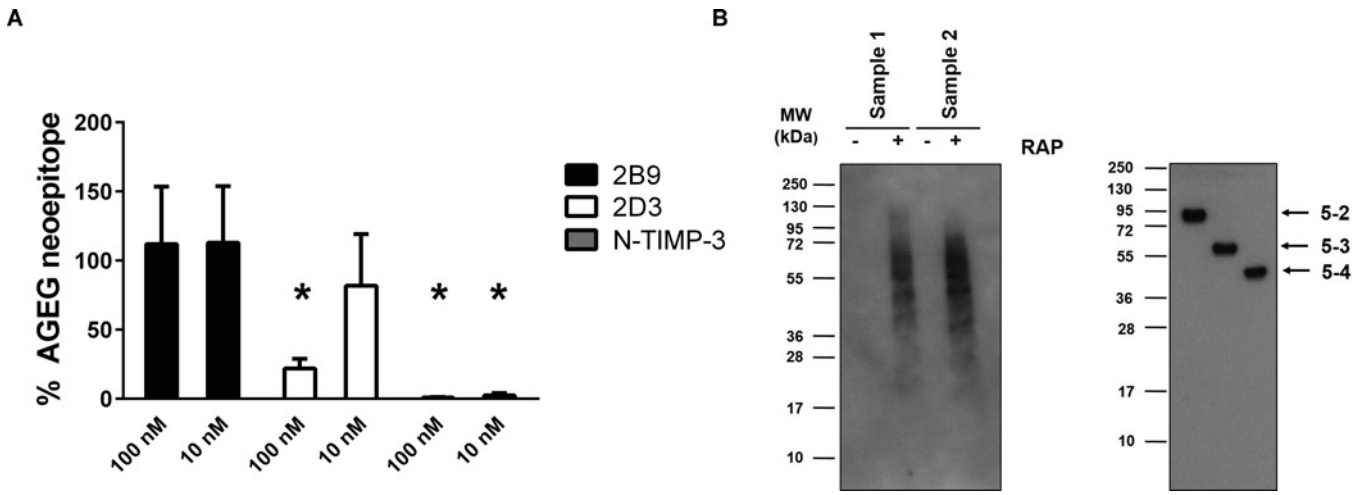


Figure 4 Effect of anti-ADAMTS-5 antibodies on aggrecan degradation by human chondrocytes

(**A**) Isolated primary chondrocytes were cultured in the presence of aggrecan (100 μg/ml) in a 24-well plate in the presence and absence of anti-ADAMTS-5 antibodies or N-TIMP-3. After 24-h incubation, the media were collected, deglycosylated in the presence of EDTA, precipitated with acetone and re-suspended in SDS/PAGE sample buffer. Samples were then subjected to Western blot and membrane was probed with polyclonal neoepitope antibody recognizing cleavage at the E^{1790}–A^{1791} site. Data are the mean $\pm$ S.E.M (n=3). *$P < 0.05$ compared with non-inhibitory antibody 1B3. (**B**) Detection of endogenous ADAMTS-5 in the conditioned medium of cultured human chondrocytes. Chondrocytes were incubated for 48 h in the presence and absence of RAP (500 nM). Media were collected, supplemented with protease inhibitor cocktail and metzincin inhibitor CT-1746, concentrated and subjected to Western blotting analysis under non-reducing conditions. The in-house antibody specific for ADAMTS-5 Cat domain 2A11 was used as a detection antibody. For comparison, detection of purified recombinant ADAMTS5-2, 5-3 and 5-4 (2 ng/lane) by 2A11 is shown.

N-TIMP-3 mapped the binding site of 2D3 in the Dis domain which is located on the prime side of the active site (the sites that interact with substrate residues located at the C-terminal side of the scissile bond). Since the peptide substrate Abz-TESE↓SRGAIY-Dpa-KK has eight residues on the prime side, sufficiently long to potentially interact with the Dis domain, we hypothesize that 2D3 is able to interfere with the binding of this peptide substrate. The binding site of 2D11 in the Cat/Dis domains is distinct from that of 2D3 and it is a weaker inhibitor. It blocks the ADAMTS5-2 activity on QF peptide and on *gst*-IGD-*flag*, but it does not block the enzyme activity on aggrecan at both the E^{392}–A^{393} and the E^{1790}–A^{1791} sites. One possible explanation for this seemingly contradictory observation is that 2D11 binds to the Cat/Dis domains loosely, but sufficient to block the hydrolysis of unglycosylated peptides or proteins. However, the weak affinity fails to inhibit the activity on native aggrecan, which makes multiple interactions with the enzyme through TS1, CysR and Sp domains [3]. In fact, the K_m values of aggrecan for ADAMTS5-2 is much lower ($K_m = 4\ \mu$M) than that of the QF peptide ($K_m > 100\ \mu$M; Salvatore Santamaria and Hideaki Nagase, unpublished work). This makes 2D11 a less efficient inhibitor when aggrecan is the substrate.

Antibody 2B9 binds to the Sp domain and inhibits the ADAMTS5-2 activity only when aggrecan or recombinant *gst*-IGD-*flag* is the substrate. The CysR and the Sp domains of aggrecanolytic ADAMTSs are responsible for effective aggrecanolytic activity. Deletion of the Sp domain from ADAMTS-5 severely affects aggrecan cleavage at E^{392}–A^{393} (50 % reduction) in the IGD region and at the E^{1499}–A^{1500} bond in the chondroitin sulfate attachment region (>99 % reduction), but it does not affect the activity against *S*-carboxmethylated transferrin, a general protease substrate [3]. Similarly, this deletion reduces the activity on unglycosylated *gst*-IGD-*flag* by 90 %, indicating that the Sp domain does not require sulfated GAGs for its effective action on aggrecan. We previously reported that the CysR domain of ADAMTS-5 is the main binder of heparin [3] and calcium pentosan polysulfate, a chemically sulfated xylanopyranose from beech wood [18]. Considering those observations together, we propose that the Sp domain and the CysR domains in ADAMTS-5 have distinct roles in aggrecanolysis: (i) the Sp domain forms protein–protein interactions with the core protein of aggrecan; and (ii) the CysR domain interacts with sulfated GAGs. Blocking of either interaction would result in an effective exosite inhibitor. Antibody 2B9 inhibits step (i). It exhibits more potent inhibition on *gst*-IGD-*flag* than on aggrecan, since aggrecan has a higher affinity for the enzyme through interaction between the CysR domain and the sulfated GAG chains. The inhibition of ADAMTS-5 by 2B9 may resemble that of auto-antibodies present in patients with thrombotic thrombocytopenic purpura that react with the Sp domain of ADAMTS-13 [30,31]. They inhibit cleavage of von Willebrand factor by interacting with the sequence within Y^{658}–Y^{665} of the Sp domain of ADAMTS-13 that complementarily binds to the substrate, the A2 domain of von Willebrand factor [32].

The primary structures of Sp domains of the ADAMTSs show no apparent homology to known structural motifs, but fold into a single globular domain in a jelly-roll topology with two antiparallel β-sheets that lie parallel to each other [24]. Many key residues of the Sp domains are conserved among ADAMTS proteins, particularly those that maintain the structure, but their overall sequence identity is low: only 19 % between ADAMTS-5 and ADAMTS-13; and 35 % between ADAMTS-4 and ADAMTS-5. It is also notable that the binding partners for the Sp domains and CysR domain of the two aggrecanases, ADAMTS-4 and ADAMTS-5, are different in that the Sp domain of ADAMTS-4 binds to sulfated GAGs [27,28]. While this introduces further complexity about the domain functions of the ADAMTSs, it supports the concept of searching for exosite inhibitors of multi-domain proteases to achieve selective substrate-dependent inhibition.

ADAMTS-5 has received much attention from researchers investigating the pathogenesis of OA. A mouse model of OA has

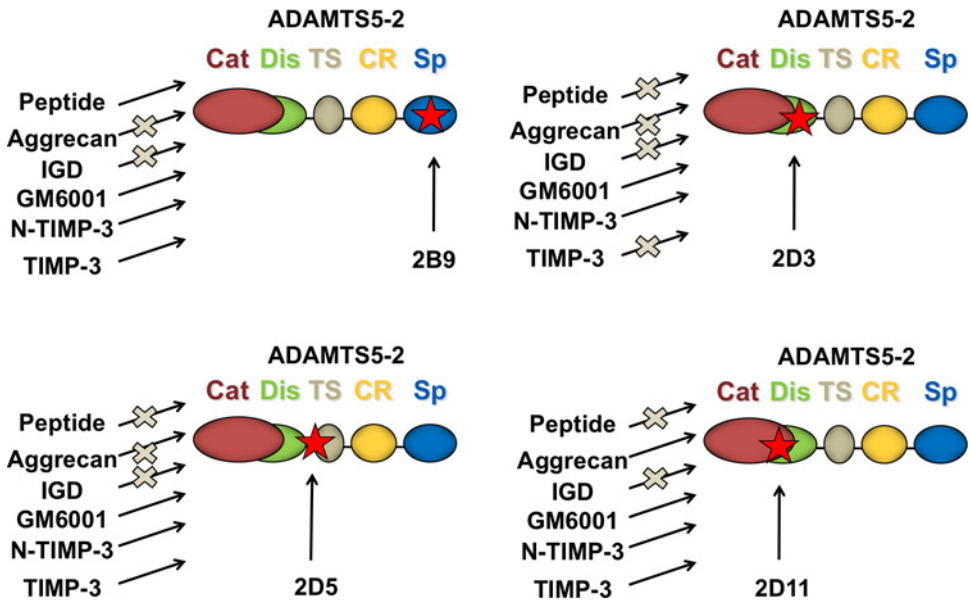


Figure 5 Interactions between inhibitory antibodies and ADAMTS-5 domains

A red star indicates the location of antibody epitope and an open cross indicates blockade of the molecular interaction resulting from antibody binding.

indicated that ADAMTS-5 is the major aggrecanase [5], but there remains a debate about which of the ADAMTSs, ADAMTS-1, -4 and/or -5, are key aggrecanase(s) in the development of human OA [33,34]. Our results support the specificity of anti-ADAMTS-5 antibodies in blocking the ADAMTS-5 activity and agree with a very recent report [35] showing a major involvement of ADAMTS-5 in endogenous aggrecan degradation. Those authors [35] reported that inhibition of ADAMTS-5 by an antibody directed against the Cat/Dis domain preferentially blocked the degradation of aggrecan in human cartilage and in cynomolgus monkeys, thus showing the efficacy of antibodies directed against these domains of ADAMTS-5. Therefore, ADAMTS-5 antibodies may be useful in the development of anti-aggrecanolytic therapies to abrogate the destructive aspects of OA. On the other hand, Chiusaroli et al. [36] reported that an anti-Sp antibody for ADAMTS-5 protected the disease progression in a murine model of spontaneous OA when injected intra-articularly, but did not report the efficacy in human cartilage. As shown in Figure 4B, in human chondrocyte culture ADAMTS-5 is C-terminally processed. Intermolecular autocatalytic cleavage in the Sp domain has been reported for ADAMTS-4 and -5 [37,38] and in the CysR domain of ADAMTS-4 [38] and other proteases are also likely to be involved in C-terminal truncation. C-terminal truncation may represent an important process for post-translational regulation of ADAMTS-5 activity *in vivo* and could potentially nullify the inhibition by antibodies directed against distal ancillary domains.

Besides pathological function, recent studies have suggested that ADAMTS-5 is important developmentally and physiologically [1]. During development it is expressed in brain, developing nerves, limbs, skeletal muscle and heart and in adult tissues it is expressed in arterial smooth muscle cells in bronchi, pancreatic ducts, glomerular mesenchymal cells in kidney, dorsal root ganglia and Schwann cells of the peripheral and autonomic nervous systems [39]. Gene ablation and transgenic mouse studies have shown that it plays a role in cardiac valve development [40], regression of the interdigital web during mouse limb morphogenesis along with ADAMTS-9 and ADAMTS-20 [41], control of fibroblast–myofibroblast transition [42], skin excision wound healing [43] and formation of multinucleated myotubes [44]. In many cases, the enzyme functions as a versican- and an aggrecan-degrading enzyme and the activity must be controlled temporally and spatially. Nevertheless, the tissues often express other related ADAMTSs. For example, ADAMTS-4 and -5 are expressed in neuronal cells and recombinant ADAMTS-4 and -5 promote neurite outgrowth *in vitro* [45,46], but it is not known which enzyme plays the major role *in vivo*. The four inhibitory antibodies described in the present work will be valuable tools to determine both physiological and pathological functions of ADAMTS-5 as they not only inhibit the target enzyme in a substrate dependent manner, but can also identify which isoforms of the enzyme are present in the tissue.

AUTHOR CONTRIBUTION

Salvatore Santamaria designed and performed the experiments, analysed the data and wrote the manuscript. Kazuhiro Yamamoto, Kenneth Botkjaer and Christopher Tape performed the experiments and analysed the data. Michael Dyson and John McCafferty generated the phage display library and the expression vectors. Gillian Murphy and Hideaki Nagase designed the experiments, analysed the data and wrote the manuscript.

ACKNOWLEDGEMENTS

We thank Dr Rob Visse for his assistance in ADAMTS-5/TIMP-3 modelling and Dr Ngee Han Lim (Kennedy Institute of Rheumatology, Oxford) for his provision of the recombinant IGD substrate and N-TIMP-3 expressed in *E. coli*.

FUNDING

This work was supported by the National Institute of Health [grant number AR40994]; the Arthritis Research UK [grant number 20563 (to K.Y.)]; the Cancer Research UK [grant

number C100/A8243 (to G.M.)]; and the Danish Cancer Society [grant number R40-A1838-11-S2 (to K.B.)].

REFERENCES

1 Nagase, H. (2012) The ADAMTS family of metalloproteinases. In Extracellular Matrix: Pathobiology And Signaling (Karamanos, N.K., ed.), p. 315-342, Walter de Gruyter GmbH & Co. KG, Berlin/Boston
2 Pratta, M.A., Yao, W., Decicco, C., Tortorella, M.D., Liu, R.Q., Copeland, R.A., Magolda, R., Newton, R.C., Trzaskos, J.M. and Arner, E.C. (2003) Aggrecan protects cartilage collagen from proteolytic cleavage. J. Biol. Chem. **278**, 45539–45545 CrossRef PubMed
3 Gendron, C., Kashiwagi, M., Lim, N.H., Enghild, J.J., Thogersen, I.B., Hughes, C., Caterson, B. and Nagase, H. (2007) Proteolytic activities of human ADAMTS-5. Comparative studies with ADAMTS-4. J. Biol. Chem. **282**, 18294-18306 CrossRef
4 Glasson, S.S., Askew, R., Sheppard, B., Carito, B., Blanchet, T., Ma, H.L., Flannery, C.R., Peluso, D., Yang, Z., Majumdar, M.K. et al. (2005) Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. Nature **434**, 644-648 CrossRef
5 Stanton, H., Rogerson, F.M., East, C.J., Golub, S.B., Lawlor, K.E., Meeker, C.T., Little, C.B., Last, K., Farmer, P.J., Campbell, I.K. et al. (2005) ADAMTS-5 is the major aggrecanase in mouse cartilage *in vivo* and *in vitro*. Nature **434**, 648-652 CrossRef
6 Coussens, L.M., Fingleton, B. and Matrisian, L.M. (2002) Matrix metalloproteinase inhibitors and cancer: trials and tribulations. Science **295**, 2387-2392 CrossRef
7 Gomis-Rüth, F.X. (2009) Catalytic domain architecture of metzincin metalloproteases. J. Biol. Chem. **284**, 15353-15357 CrossRef
8 Nuti, E., Santamaria, S., Casalini, F., Yamamoto, K., Marinelli, L., La Pietra, V., Novellino, E., Orlandini, E., Nencetti, S., Marini, A.M. et al. (2013) Arylsulfonamide inhibitors of aggrecanases as potential therapeutic agents for osteoarthritis: synthesis and biological evaluation. Eur. J. Med. Chem. **62**, 379-394 CrossRef
9 Saghatelian, A., Jessani, N., Joseph, A., Humphrey, M. and Cravatt, B.F. (2004) Activity-based probes for the proteomic profiling of metalloproteases. Proc. Natl. Acad. Sci. U.S.A. **101**, 10000-10005 CrossRef
10 Hardy, J.A. and Wells, J.A. (2004) Searching for new allosteric sites in enzymes. Curr. Opin. Struct. Biol. **14**, 706-715 CrossRef
11 Nagase, H. and Fushimi, K. (2008) Elucidating the function of non catalytic domains of collagenases and aggrecanases. Connect. Tissue Res. **49**, 169-174 CrossRef
12 Troeberg, L., Mulloy, B., Ghosh, P., Lee, M.H., Murphy, G. and Nagase, H. (2012) Pentosan polysulfate increases affinity between ADAMTS-5 and TIMP-3 through formation of an electrostatically driven trimolecular complex. Biochem. J. **443**, 307-315 CrossRef
13 Hascall, V.C. and Sajdera, S.W. (1969) Protein-polysaccharide complex from bovine nasal cartilage. The function of glycoprotein in the formation of aggregates. J. Biol. Chem. **244**, 2384–2396 PubMed
14 Manning, H.B., Nickdel, M.B., Yamamoto, K., Lagarto, J.L., Kelly, D.J., Talbot, C.B., Kennedy, G., Dudhia, J., Lever, J., Dunsby, C. et al. (2013) Detection of cartilage matrix degradation by autofluorescence lifetime. Matrix Biol. **32**, 32–38 CrossRef PubMed
15 Wayne, G.J., Deng, S.J., Amour, A., Borman, S., Matico, R., Carter, H.L. and Murphy, G. (2007) TIMP-3 inhibition of ADAMTS-4 (aggrecanase-1) is modulated by interactions between aggrecan and the C-terminal domain of ADAMTS-4. J. Biol. Chem. **282**, 20991–20998 CrossRef PubMed
16 Knight, C.G., Willenbrock, F. and Murphy, G. (1992) A novel coumarin-labelled peptide for sensitive continuous assays of the matrix metalloproteinases. FEBS Lett. **296**, 263–266 CrossRef PubMed
17 Lim, N.H., Kashiwagi, M., Visse, R., Jones, J., Enghild, J.J., Brew, K. and Nagase, H. (2010) Reactive-site mutants of N-TIMP-3 that selectively inhibit ADAMTS-4 and ADAMTS-5: biological and structural implications. Biochem. J. **431**, 113–122 CrossRef PubMed
18 Troeberg, L., Fushimi, K., Khokha, R., Emonard, H., Ghosh, P. and Nagase, H. (2008) Calcium pentosan polysulfate is a multifaceted exosite inhibitor of aggrecanases. FASEB J. **22**, 3515–3524 CrossRef PubMed
19 Schofield, D.J., Pope, A.R., Clementel, V., Buckell, J., Chapple, S.D., Clarke, K.F., Conquer, J.S., Crofts, A.M., Crowther, S.R., Dyson, M.R. et al. (2007) Application of phage display to high throughput antibody generation and characterization. Genome Biol. **8**, R254 CrossRef PubMed
20 Falk, R., Falk, A., Dyson, M.R., Melidoni, ,A.N., Parthiban, K., Young, J.L., Roake, W. and Mc Cafferty, J. (2012) Generation of anti-Notch antibodies and their application in blocking Notch signalling in neural stem cells. Methods **58**, 69-78 CrossRef
21 Mosyak, L., Georgiadis, K., Shane, T., Svenson, K., Hebert, T., McDonagh, T., Mackie, S., Olland, S, Lin, L., Zhong, X. et al. (2008) Crystal structures of the two major aggrecan degrading enzymes, ADAMTS4 and ADAMTS5. Protein Sci. **17**, 16–21 CrossRef PubMed
22 Wisniewska, M., Goettig, P., Maskos, K., Belouski, E., Winters, D., Hecht, R., Black, R. and Bode, W. (2008) Structural determinants of the ADAM inhibition by TIMP-3: crystal structure of the TACE–N-TIMP-3 complex. J. Mol. Biol. **381**, 1307–1319 CrossRef PubMed
23 Gomis-Rüth, F.X., Maskos, K., Betz, M., Bergner, A., Huber, R., Suzuki, K., Yoshida, N., Nagase, H., Brew, K., Bourenkov, G.P. et al. (1997) Mechanism of inhibition of the human matrix metalloproteinase stromelysin-1 by TIMP-1. Nature **389**, 77–81 CrossRef PubMed
24 Akiyama, M., Takeda, S., Kokame, K., Takagi, J. and Miyata, T. (2009) Crystal structures of the noncatalytic domains of ADAMTS13 reveal multiple discontinuous exosites for von Willebrand factor. Proc. Natl. Acad. Sci. U.S.A. **106**, 19274–19279 CrossRef PubMed
25 Schechter, I. and Berger, A. (1967) On the size of the active site in proteases. Biochem. Biophys. Res. Commun. **27**, 157-162 CrossRef
26 Brew, K. and Nagase, H. (2010) The tissue inhibitors of metalloproteinases (TIMPs): an ancient family with structural and functional diversity. Biochim. Biophys. Acta **1803**, 55-71 CrossRef
27 Fushimi, K., Troeberg, L., Nakamura, H., Lim, N.H. and Nagase, H. (2008) Functional differences of the catalytic and non-catalytic domains in human ADAMTS-4 and ADAMTS-5 in aggrecanolytic activity. J. Biol. Chem. **283**, 6706-6716 CrossRef
28 Kashiwagi, M., Enghild, J.J., Gendron, C., Hughes, C., Caterson, B., Itoh, Y. and Nagase, H. (2004) Altered proteolytic activities of ADAMTS-4 expressed by C-terminal processing. J. Biol. Chem. **279**, 10109-10119 CrossRef
29 Yamamoto, K., Troeberg, L., Scilabra, S.D., Pelosi, M., Murphy, C.L., Strickland, D.K. and Nagase, H. (2013) LRP-1-mediated endocytosis regulates extracellular activity of ADAMTS5 in articular cartilage. FASEB J. **27**, 511-521 CrossRef
30 Klaus, C., Plaimauer, B., Studt, J.D., Dorner, F., Lammle, B., Mannucci, P.M. and Scheiflinger, F. (2004) Epitope mapping of ADAMTS13 autoantibodies in acquired thrombotic thrombocytopenic purpura. Blood **103**, 4514-4519 CrossRef
31 Luken, B.M., Turenhout, E.A., Hulstein, J.J., Van Mourik, J.A., Fijnheer, R. and Voorberg, J. (2005) The spacer domain of ADAMTS13 contains a major binding site for antibodies in patients with thrombotic thrombocytopenic purpura. Thromb. Haemost. **93**, 267–274 PubMed
32 Pos, W., Crawley, J.T., Fijnheer, R., Voorberg, J., Lane, D.A. and Luken, B.M. (2010) An autoantibody epitope comprising residues R660, Y661, and Y665 in the ADAMTS13 spacer domain identifies a binding site for the A2 domain of VWF. Blood **115**, 1640-1649 CrossRef
33 Naito, S., Shiomi, T., Okada, A., Kimura, T., Chijiiwa, M., Fujita, Y., Yatabe, T., Komiya, K., Enomoto, H., Fujikawa, K. et al. (2007) Expression of ADAMTS4 (aggrecanase-1) in human osteoarthritic cartilage. Pathol. Int. **57**, 703-711 CrossRef
34 Song, R.H., Tortorella, M.D., Malfait, A.M., Alston, J.T., Yang, Z., Arner, E.C. and Griggs, D.W. (2007) Aggrecan degradation in human articular cartilage explants is mediated by both ADAMTS-4 and ADAMTS-5. Arthritis Rheum. **56**, 575-585 CrossRef
35 Larkin, J., Lohr, T.A., Elefante, L., Shearin, J., Matico, R., Su, J.L., Xue, Y., Liu, F., Genell, C., Miller, R.E. et al. (2015) Translational development of an ADAMTS-5 antibody for osteoarthritis disease modification. Osteoarthritis Cartilage **23**, 1254–1266 CrossRef PubMed
36 Chiusaroli, R., Visentini, M., Galimberti, C., Casseler, C., Mennuni, L., Covaceuszach, S., Lanza, M., Ugolini, G., Caselli, G., Rovati, L.C. et al. (2013) Targeting of ADAMTS-5's ancillary domain with the recombinant mAb CRB0017 ameliorates disease progression in a spontaneous murine model of osteoarthritis. Osteoarthritis Cartilage **21**, 1807-1810 CrossRef
37 Flannery, C.R., Zeng, W., Corcoran, C., Collins-Racie, L.A., Chockalingam, P.S., Hebert, T., Mackie, S.A., McDonagh, T., Crawford, T.K, Tomkinson, K.N. et al. (2002) Autocatalytic cleavage of ADAMTS-4 (aggrecanase-1) reveals multiple glycosaminoglycan-binding sites. J. Biol. Chem. **277**, 42775–42880 CrossRef PubMed
38 Georgiadis, K., Crawford, T., Tomkinson, K., Shakey, Q., Stahl, M., Morris, E., Collins-Racie, L.A. and LaVallie, E. (2002) ADAMTS-5 is autocatalytic at a E753-G754 site in the spacer domain. Trans. Orthop. Res. Soc. **48**, 167
39 McCulloch, D.R., Le Goff, C., Bhatt, S., Dixon, L.J., Sandy, J.D. and Apte, S.S. (2009) Adamts5, the gene encoding a proteoglycan-degrading metalloprotease, is expressed by specific cell lineages during mouse embryonic development and in adult tissues. Gene Expr. Patterns **9**, 314-323 CrossRef
40 Dupuis, L.E., McCulloch, D.R., McGarity, J.D., Bahan, A., Wessels, A., Weber, D., Diminich, A.M., Nelson, C.M., Apte, S.S. and Kern, C.B. (2011) Altered versican cleavage in ADAMTS5 deficient mice; a novel etiology of myxomatous valve disease. Dev. Biol. **357**, 152-164 CrossRef
41 McCulloch, D.R., Nelson, C.M., Dixon, L.J., Silver, D.L., Wylie, J,D., Lindner, V., Sasaki, T., Cooley, M.A., Argraves, W.S. and Apte, S.S. (2009) ADAMTS metalloproteases generate active versican fragments that regulate interdigital web regression. Dev. Cell **17**, 687-698 CrossRef
42 Hattori, N., Carrino, D.A., Lauer, M.E., Vasanji, A., Wylie, J.D., Nelson, C.M. and Apte, S.S. (2011) Pericellular versican regulates the fibroblast-myofibroblast transition: a role for ADAMTS5 protease-mediated proteolysis. J. Biol. Chem. **286**, 34298-34310 CrossRef

43 Velasco, J., Li, J., DiPietro, L., Stepp, M.A, Sandy, J.D. and Plaas, A. (2011) ADAMTS5 ablation blocks murine dermal repair through CD44-mediated aggrecan accumulation and a switch in TGFb1 signaling from ALK5 to ALK1. J. Biol. Chem. **286**, 26016-26027 CrossRef

44 Stupka, N., Kintakas, C., White, J.D., Fraser, F.W., Hanciu, M, Aramaki-Hattori, N., Martin, S., Coles, C., Collier, F., Ward, A.C. et al. (2013) Versican processing by a disintegrin-like and metalloproteinase domain with thrombospondin-1 repeats proteinases-5 and -15 facilitates myoblast fusion. J. Biol. Chem. **288**, 1907–1917 CrossRef PubMed

45 Hamel, M.G., Ajmo, J.M., Leonardo, C.C., Zuo, F., Sandy, J.D. and Gottschall, P.E. (2008) Multimodal signaling by the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) promotes neurite extension. Exp. Neurol. **210**, 428-440 CrossRef

46 Cua, R.C., Lau, L.W., Keough, M.B., Midha, R., Apte, S.S. and Yong, V.W. (2013) Overcoming neurite inhibitory chondroitin sulfate proteo glycans in the astrocyte matrix. Glia **61**, 972–984 CrossRef PubMed

Received 6 July 2015/21 August 2015; accepted 24 August 2015
Accepted Manuscript online 24 August 2015, doi:10.1042/BJ20150758