The low binding affinity of ADAMTS4 for citrullinated fibronectin may contribute to the destruction of joint cartilage in rheumatoid arthritis

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Abstract

Objectives

Rapid cartilage degradation in the joints is observed in rheumatoid arthritis (RA). ADAMTS4 (a disintegrin and metalloproteinase with thrombospondin motifs-4) degrades aggrecan, the primary component of cartilage, therefore contributing to joint erosion in RA. The proteolytic activity of ADAMTS4 is inhibited by fibronectin (FN). FN is abundantly expressed in the synovia in RA and is modified by citrullination, the conversion of peptidylarginine to citrulline. This study aims to investigate the binding ability of citrullinated FN (cFN) to ADAMTS4 and the effect of cFN on aggrecanase activity.

Methods

The full-length recombinant ADAMTS4 was purified from HEK293 cells that were transiently transfected with a full-length cDNA coding for human ADAMTS4. A 40-kDa FN fragment exhibiting heparin binding was citrullinised with rabbit peptidylarginine deaminase. The binding activity of the full-length recombinant ADAMTS4 to cFN was investigated in an in vitro binding assay. The proteolytic activity of ADAMTS4 after incubation with cFN was determined using an aggrecanase activity kit, in which the ARGSVIL peptide is produced by digestion with aggrecanase.

Results

cFN displayed significantly decreased binding activity with ADAMTS4 compared with FN. The full-length ADAMTS4 produced large amounts of the ARGSVIL peptide, but the amount was markedly decreased in the presence of FN. The production of this peptide approached the normal level when the full-length ADAMTS4 was incubated with cFN.

Conclusion

FN following citrullination is less effective in inhibiting the proteolytic activity of ADAMTS4. It is known that PADI4, an enzyme active in citrullination, is highly expressed in the synovial tissue in RA. Our results suggest that PADI4 in the RA synovium may contribute to cartilage destruction via the citrullination of FN.

Key words

rheumatoid arthritis, joint cartilage destruction, ADAMTS4, fibronectin, citrullination, PADI4

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Introduction

The erosion of joint cartilage is a primary feature of rheumatoid arthritis (RA) and leads to a loss of joint function over time. Cartilage destruction in arthritic joints largely depends on the breakdown of the extracellular matrix via degradation of the proteoglycan aggrecan (1). Aggrecanases, which belong to the adamalysin subfamily of metalloproteinases, promote the degradation of aggrecan by cleaving aggrecan at the Glu 373-Ala 374 site within the interglobular domain of the aggrecan protein (2). There is a growing body of evidence indicating that aggrecanase activity plays a role in the degradation of proteoglycan aggrecan under pathological conditions. Aggrecanase-1 (a disintegrin and metalloproteinase with thrombospondin motifs-4, or ADAMTS4) and aggrecanase-2 (ADAMTS5) have been identified in cartilage and are thought to play pivotal roles in the degradation of cartilage aggrecan in RA (1). Aggrecanases have also been identified in the synovial fluid of RA and osteoarthritis (OA) patients and have been implicated in the process of cartilage degradation (3, 4).

Using *in vitro* binding assays, Hashimoto *et al*. demonstrated that the aggrecanase activity of ADAMTS4 was inhibited by fibronectin (FN) through an interaction involving their C-terminal domains, and these researchers suggested that the extracellular regulatory mechanism of ADAMTS4 activity may be important in the degradation of aggrecan in arthritic cartilage (5). FN is abundantly expressed in arthritic synovia, and the metabolism of FN may contribute to cartilage damage *in vivo* (6, 7). PADI4, a member of the peptidylarginine deaminase family encoded by a gene located on chromosome 1p36, has been confirmed to confer susceptibility to RA among Japanese, Korean and German populations (8-10). This enzyme converts post-translationally modified peptidylarginine into citrulline in a process termed citrullination, which plays a profound role in the autoimmune response of RA (11). We previously detected the expression of PADI4 in RA synovial membranes and identified the enzyme in T cells, B cells, macrophages, neutrophils, fibroblast-like cells, endothelial cells, apoptotic cells and fibrin deposits within the synovial membrane (12). PADI4 was also detected in synovial fluids in RA (13). In addition, we found that PADI4 catalyses FN in the RA synovium, and high levels of citrullinated fibronectin (cFN) were present in the blood and synovial membranes of patients with RA (14). Following citrullination, many proteins change their conformation and functional activity (15). Based on the aforementioned data, we hypothesised that FN changes its binding activity to ADAMTS4 following citrullination and thus affects the proteolytic activity of the enzyme. To test this hypothesis, we investigated the binding activity of cFN to aggrecanase using *in vitro* binding assays and studied the effect of cFN on aggrecanase activity. The larger purpose of this study was to elucidate a possible mechanism for joint tissue destruction in RA.

Materials and methods

Preparation of full-length recombinant ADAMTS4

The full-length recombinant human ADAMTS4 protein was prepared by the technical service department of Origene (Maryland, USA), a US-based research-tool company that focuses on the commercial collection of full-length human cDNAs in a standard expression vector. The open reading frame (ORF) of the ADAMTS4 gene was sub-cloned into a TrueORF cDNA clone. Engineered as expression-ready clones with verified ORFs and built-in C-terminal tags, TrueORFs express proteins with tracking tags, facilitating the detection and isolation of the expressed proteins. Human HEK293 cells were transiently transfected with the ADAMTS4-containing TrueORF clone. The overexpressed recombinant ADAMTS4 protein was purified using an anti-DDK affinity column. The recombinant ADAMTS4 was eluted with 10% glycerol, 100 mM glycine, and 25 mM Tris-HCl, pH 7.3. A commercially available truncated version of the ADAMTS4 recombinant protein that starts at the catalytic domain and ends before the spacer domain (Phe213-Cys685) was obtained

from R&D Systems (Minnesota, USA). SDS-PAGE and western blot analyses were performed to determine the sizes of the full-length ADAMTS4 recombinant protein and the truncated ADAMTS4 recombinant protein lacking the C-terminus. The concentrations of the proteins were determined using BCA protein assay reagents (Thermo, Illinois, USA), and 2-μg samples of total protein were loaded and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), trans-blotted onto nylon membranes and probed with antibodies against ADAMTS4 and citrulline. Antibodies against the N-terminus, propeptide and C-terminus of the ADAMTS4 protein (Abcam, Cambridge, UK) were used in the analysis to determine the identities of the ADAMTS4 recombinant proteins. A western blotting kit (KPL, Maryland, USA) was used to detect the immuno-signals according to the manufacturer's instructions.

Citrullination of FN

The heparin-binding 40-kDa fragment of human plasma fibronectin (Millipore, Massachusetts, USA) was purified from a chymotryptic digest of human plasma fibronectin. This preparation contains the CS-1 heparin-binding region of fibronectin. Fibronectin at a final concentration of 0.1 mg/ml was incubated with 10 units/ml of peptidylarginine deaminase (PAD) from rabbit skeletal muscle (Sigma, Missouri, USA) in a working buffer containing 100 mM Tris-HCl and 5 mM $CaCl_2$ (pH 7.4) for 4 h at 37°C. The enzyme was omitted from the control reaction. Citrullination was verified by western blot analysis using an anti-citrulline antibody (Abcam) that was raised in rabbits using conjugated citrulline glutaraldehyde protein. Western blotting was performed as described above.

In vitro assay of cFN binding to ADAMTS4

The protocol for this experiment was based on a study by Hashimoto *et al*. (5). Uncitrullinated FN and citrullinated FN were diluted with 0.05 M carbonate/bicarbonate buffer (pH 9.6) to a final concentration of 50 μg/ml; aliquots were loaded into 96-well ELISA microplates and held overnight at 4°C. After washing with TNCB (50 mM Tris-HCl, pH 7.5, 0.15 mM NaCl, 10 mM $CaCl_2$, 0.05% Brij 35 and 0.02% NaN3), nonspecific binding was blocked with PBS buffer containing 1% BSA for 2 h at room temperature. Recombinant human ADAMTS4 was diluted with binding buffer (TNCB containing 2% BSA) to a concentration of 10 μg/ml and was added to the plates, which were subsequently incubated for 2 h at 37°C. Following a wash with PBST, the plates were incubated for 1 h at room temperature with the anti-ADAMTS4 propeptide domain antibody (Abcam) diluted 2.000-fold in PBS buffer containing 2% BSA. After another wash with PBST, alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma) that had been diluted 5.000-fold with PBS buffer containing 2% BSA was added to the wells followed by incubation for 1 h at room temperature. The reaction signals were developed in alkaline phosphatase yellow and measured at 405 nm. Before the experiment, a series of cFN concentrations were incubated with various concentrations of ADAMTS4 protein to establish the optimal experimental conditions. A series of controls were prepared, including incubation without cFN, the ADAMTS4 protein, and the addition of only the first or second antibody to estimate the degree of nonspecific binding. The control experiment also used the recombinant ADAMTS4 without the C-terminus.

Detection of aggrecanase activity binding to the citrullinated fibronectin

The aggrecanase activity of ADAMTS4 after binding to cFN was measured with the Aggrecanase Activity Assay Kit from Abnova (Taibei, Taiwan). This kit is used to measure the activity of aggrecanases and screen aggrecanase inhibitors. In this assay, a recombinant fragment of the human aggrecan interglobular domain (aggrecan-IGD) is digested with aggrecanase (ADAMTS4) in test samples. The proteolytic cleavage of the substrate releases an aggrecan peptide with the N-terminal sequence ARGSVIL (the ARGSVIL peptide). The yield of ARGSVIL peptide is subsequently quantified using two monoclonal anti-peptide antibodies. Following the assay procedure provided by the manufacturer, the ADAMTS4 working standard and the substrate-inhibitor mixture were prepared, and the proteolytic reaction was conducted by adding the ADAMTS4 working standard or test sample to the substrate-inhibitor mixture. The reaction was incubated for 15 min at 37°C and stopped by pipetting 150 μl of EDTA dilution buffer. The amount of the ARGSVIL peptide produced by aggrecanase is calculated based on a standard curve constructed using authentic ARGSVIL peptide.

The test samples consisted of the full-length ADAMTS4, FN, FN plus the full-length ADAMTS4, cFN, cFN plus the full-length ADAMTS4, truncated ADAMTS4, FN plus the truncated ADAMTS4 and cFN plus the truncated ADAMTS4. The final concentration of FN was 50 ug/ml, the final concentration of cFN was 50 ug/ml, the final concentration of the full-length ADAMTS4 was 10 ug/ml, and the final concentration of the truncated ADAMTS4 was 10 ug/ml. The citrullination of FN was performed as described above. ADAMTS4 was incubated with citrullinated FN as described above.

Statistical analysis of the data was performed using the SPSS V.16 software package (SPSS, USA). Median differences were tested with the Mann-Whitney U-test. *p*-values of less than 0.05 were considered to be significant. When three groups were compared, a Kruskal-Wallis test was first conducted.

Results

The heparin-binding 40-kDa fragment of human plasma fibronectin was treated with rabbit PAD. The citrullination of the fibronectin was verified by western blot analysis using an anti-citrulline antibody. The immuno-signal of citrulline was detected in FN following the treatment of PAD, but not in the absence of PAD treatment, thereby indicating that FN was citrullinised by PAD. The results are illustrated in Figure 1.

Recombinant ADAMTS4 that was encoded by an intact ORF was expressed in HEK293 cells, and the ADAMTS4 protein was purified from the cell line.

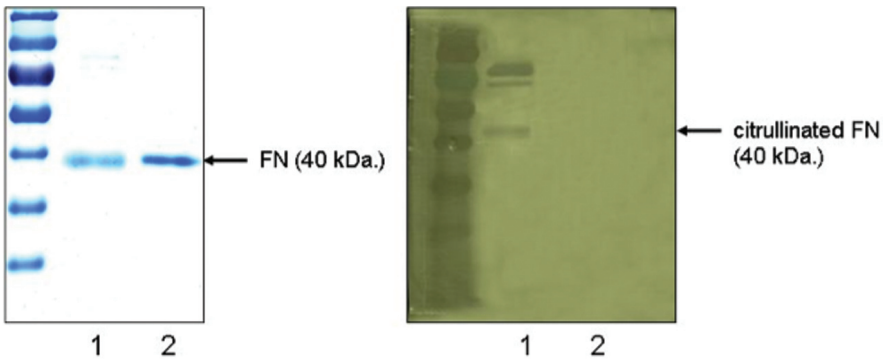

Fig. 1. SDS-PAGE and western blot analysis of the heparin-binding 40-kDa fragment of human plasma fibronectin. (**A**) A band of 40 kDa was detected by SDS-PAGE, as expected in the FN with PAD treatment (lane 1) and FN without PAD treatment (lane 2) conditions. (**B**) The immuno-signal of citrullination was detected in PAD-treated FN (lane 1) by western blot analysis using an anti-citrulline antibody, but was not observed in untreated FN, confirming that FN was citrullinised by the PAD treatment.

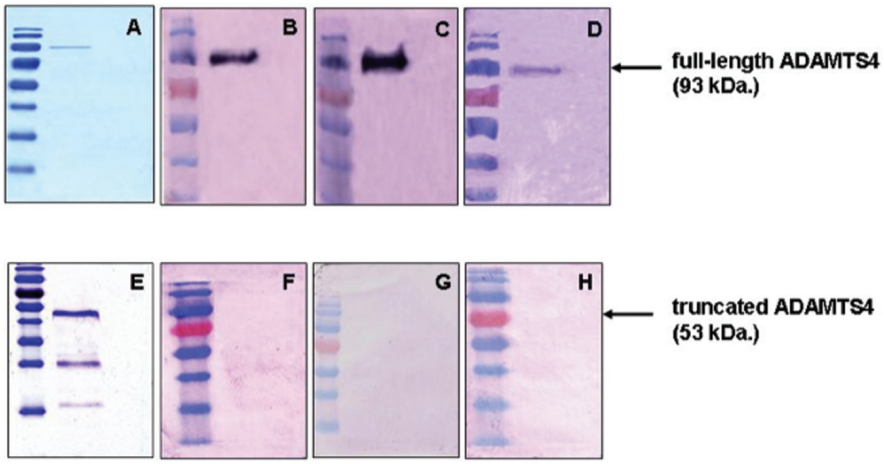

Fig. 2. SDS-PAGE and western blot analysis of the recombinant full-length and truncated ADAMTS4 proteins. (**A**) A band of 93 kDa was detected by SDS-PAGE in the full-length ADAMTS4 samples and was identified by western blot analysis using (**B**) an anti-ADAMTS4 antibody against the N-terminus, (**C**) an anti-ADAMTS4 antibody against the propeptide domain and (**D**) an anti-ADAMTS4 antibody against the C-terminus. These results indicate that this ADAMTS4 recombinant protein had the expected full molecular weight and length. (**E**) A band of 53 kDa was detected in the truncated ADAMTS4 samples by SDS-PAGE, but not recognised by western blot analysis using (**F**) the antibody against the N-terminus, (**G**) the anti-ADAMTS4 antibody against the propeptide-domain or (**H**) the antibody against the C-terminus. These results indicate that this short form of the ADAMTS4 recombinant protein did not contain the N-terminus or the C-terminus, as expected.

A band of 93 kDa was observed in SDS-PAGE, and this band was recognised by western blotting with anti-ADAMTS4 antibodies against the enzyme's N-terminus, propeptide domain and C-terminus, indicating that this ADAMTS4 recombinant protein was of the correct molecular weight and, therefore, was full-length (Fig. 2). A truncated ADAMTS4 recombinant protein that lacked the C-terminus was also examined using SDS-PAGE and western blot analysis. A band of 53 kDa was observed in SDS-PAGE, but this band was not recognised by anti-ADAMTS4 antibodies against the N-terminus, the propeptide domain or the C-terminus, indicating that the truncated ADAMTS4 recombinant protein started at the catalytic domain and ended before the spacer domain, as indicated by the manufacturer (Fig. 2).

To determine the effect of citrullination on the interaction of ADAMTS4 with FN, a binding assay was performed by incubating the recombinant ADAMTS4 protein with FN that was either citrullinated or not. A high absorbance at 405 nm was detected when uncitrullinated FN was incubated with the full-length ADAMTS4 protein, indicating the strong binding affinity of FN for the enzyme. On the other hand, the absorbance significantly declined when citrullinated FN was incubated with recombinant ADAMTS4, indicating the low binding affinity of cFN for the enzyme. Additionally, the recombinant ADAMTS4 protein with a truncation at the C-terminus displayed low absorbance at 405 nm when the enzyme was incubated with both FN and cFN, indicating that either FN or cFN did not show binding ability with the truncated ADAMTS4. The results of the *in vitro* binding assay are presented in Figure 3. The experiment was repeated three times, yielding notably similar results each time.

To determine the proteolytic activity of ADAMTS4 in the presence of cFN, an aggrecanase activity assay was performed using an Aggrecanase Activity Assay Kit. A recombinant fragment of the human aggrecan interglobular domain was digested by ADAMTS4 in the test sample. The concentration of the ARGSVIL peptide, which corresponded to the aggrecanase activity of ADAMTS4, was obtained by measuring absorbance at 450 nm in the proteolytic reaction based on the standard curve. The reactions containing FN or cFN without ADAMTS4 produced small amounts of the ARGSVIL peptide at concentrations of 0.2269±0.0224 nM and 0.2122±0.0032 nM, respectively. The reaction containing the full-length ADAMTS4 yielded a large amount of the ARGSVIL peptide at a concentration of 0.9079±0.0071 nM, but the production markedly decreased to 0.5739±0.018 nM when the full-length ADAMTS4 was first incubated with FN. The production returned to a near-normal level, a concentration of 0.83±0.0202 nM, when the full-length ADAMTS4 was incubated with cFN. In addition, the reaction containing the truncated ADAMTS4 without FN or cFN yielded the highest concentration of ARGSVIL peptide, 36.42±3.672 nM, which was almost 40 times higher than the full-length ADAMTS4. Peptide production was not significantly altered when FN (41.09±1.01 nM) or cFN (40.6±2.782 nM) were added to the reaction. These results indicate that the truncated ADAMTS4 without the C-terminus does not bind FN or cFN (Fig. 4).

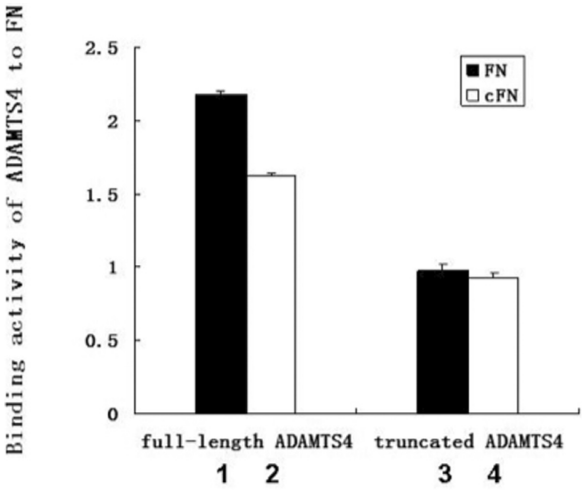

Fig. 3. A binding activity assay to characterise the interactions of recombinant ADAMTS4 with citrullinated (cFN) and uncitrullinated FN. Lane 1 shows the binding activity of FN with full-length ADAMTS4, lane 2 shows the binding affinity of cFN with full-length ADAMTS4, lane 3 shows the binding affinity of FN with ADAMTS4 lacking the C-terminus, and lane 4 shows the binding affinity of cFN with ADAMTS4 lacking the C-terminus. The binding activities were measured by the absorbance of the reactions at 405 nm and are expressed as the mean±SD.

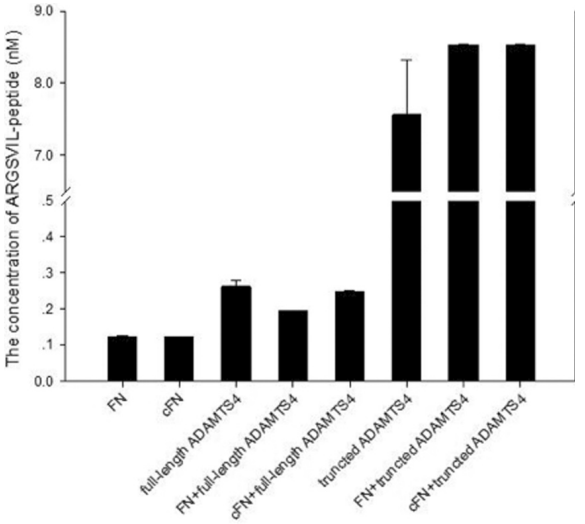

Fig. 4. An aggrecanase activity assay for ADAMTS4 binding to citrullinated fibronectin. The proteolytic activity of ADAMTS4 was determined by measuring the concentration of the ARGSVIL peptide. After incubation with FN, the full-length ADAMTS4 showed low activity in digesting aggrecan-IGD compared with the reaction containing the full-length ADAMTS4 alone. ARGSVIL peptide production returned to the normal level when ADAMTS4 was incubated with cFN.

Discussion

Using chemical cross-linking reactions, yeast two-hybrid assays and solid-phase binding assays, Hashimoto *et al*. found that the C-terminal domain of FN is capable of binding to the C-terminal spacer domain of ADAMTS4. They also reported that the aggrecanase activity of ADAMTS4 was dose-dependently inhibited by FN and suggested that the aggrecanase activity of ADAMTS4 is inhibited by FN through the interaction of their C-terminal domains and that an extracellular regulatory mechanism of ADAMTS4 activity is involved in the degradation of aggrecan in arthritic cartilage (5). FN fragments are abundant in the cartilage and synovium of RA and OA joints and have been shown to promote cartilage catabolism during the progression of arthritic diseases (6, 7). We previously found that FN was significantly modified by citrullination in synovial membranes in RA, and we also detected high levels of citrullinated FN in the blood of RA patients (14). In the present study, we found that the binding affinity of ADAMTS4 to citrullinated FN was significantly decreased compared with its binding affinity to uncitrullinated FN. Conversely, ADAMTS4 had stronger proteolytic activity in presence of cFN than the enzyme in the presence of FN, which corresponds to the results of the *in vitro* binding assay. The abovementioned result suggests that citrullination may change the conformation of FN and thus decrease its ability to bind ADAMTS4, consequently releasing the proteolytic activity of the enzyme to degrade aggrecan under physiological conditions. Because PADI4 displays increased expression in the synovial membranes and fluids of RA joints (12, 13), this enzyme may suppress the inhibitory effect of FN on ADAMTS4 by citrullination and is ultimately involved in the destruction of RA joint cartilage. Because PADI4 also has low expression in the normal tissues (12), effect of cFN on ADAMTS4 activity may be one of regulatory mechanisms on the cartilage destruction in RA. In addition, we also detected decreased aggrecanase activity of ADAMTS4 in the presence of FN, which confirms the findings of Hashimoto *et al*.

Peters *et al*. detected FN isoforms ranging from approximately 47 to over 200 kDa in both RA and OA synovial fluid by gelatin affinity isolation and 2D western blotting (16). Hashimoto *et al*. found that an FN fragment with a molecular weight of 40 kDa completely blocked the activity of wild-type ADAMTS4, whereas an FN fragment with molecular weight 120 kDa inhibited only 30% of the activity (5). In our study, we used the heparin-binding 40-kDa fragment of human plasma FN that was purified from a chymotryptic digest of human plasma FN. Our results demonstrate that this FN fragment has a high binding affinity for the full-length ADAMTS4 protein, consistent with the findings of Hashimoto *et al*. It is possible that other, possibly larger, FN fragments, in addition to the 40 kDa fragment, also display specific binding to ADAMTS4 following citrullination in physiological condition.

To examine the mode of activation of ADAMTS4, Gao *et al*. transfected JJ012 cells (a human chondrosarcoma cell line) with the full-length cDNA of human ADAMTS4 and obtained a high-molecular-weight form of the enzyme (p100) and three truncated forms (p75, p60 and p50). Aggrecanase assays revealed that only the p60 and p50 forms (with C-terminal truncations) exhibited strong aggrecanase activity, thereby suggesting that the *in vivo* production of proteolytically active ADAMTS4 requires the removal of the C-terminal spacer domain. In addition, these authors detected the p75, p60, and p50 forms of ADAMTS4 in both pig synovia and human cartilage (17). Although there are no reports of the 93-kDa form of ADAMTS4 acting as a zymogen form of the enzyme

before intracellular processing, Hashimoto *et al*. confirmed that a 93-kDa recombinant ADAMTS4 had aggrecanase activity. The binding of the full-length ADAMTS4 to FN may exert an intracellular inhibitory effect on aggrecanase activity (5). Using a series of domain deletion mutants, Kashiwagi *et al*. found that ADAMTS4 with a deletion of the C-terminal spacer domain showed strong activity in hydrolysing both the Glu373-Ala374 and Glu1480-Gly1481 bonds of aggrecan (18). In the present study, both citrullinated and uncitrullinated FN always exhibited low binding activity with the truncated ADAMTS4, indicating that the C-terminus of ADAMTS4 plays an important role in mediating the binding activity of the enzyme to FN. It is possible that both the truncated and full-length forms of ADAMTS4 that are regulated by FN are involved in the degradation of aggrecan in the arthritic joint.

In summary, our experiments revealed that following citrullination, FN binding to ADAMTS4 is decreased, liberating the aggrecanase activity of ADAMTS4, thus suggesting that high expression of PADI4 may decrease the inhibition of ADAMTS4 by FN citrullination. Although an *in vitro* assay of FN binding to ADAMTS4 is an insufficient method to demonstrate specific binding definitively under physiological conditions, these results provide a better understanding of the pathogenic process of joint destruction in RA.

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