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Inhibition of ADAMTS-7 and ADAMTS-12 degradation of cartilage oligomeric matrix protein by alpha-2-macroglobulin

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Summary

Objective: As we previously reported, ADAMTS-7 and ADAMTS-12, two members of ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs) family, degrade cartilage oligomeric matrix protein (COMP) *in vitro* and are significantly induced in the cartilage and synovium of arthritic patients [Liu CJ, Kong W, Ilalov K, Yu S, Xu K, Prazak L, *et al.* ADAMTS-7: a metalloproteinase that directly binds to and degrades cartilage oligomeric matrix protein. FASEB J 2006;20(7):988–90; Liu CJ, Kong W, Xu K, Luan Y, Ilalov K, Sehgal B, *et al.* ADAMTS-12 associates with and degrades cartilage oligomeric matrix protein. J Biol Chem 2006;281(23):15800–8]. The purpose of this study was to determine (1) whether cleavage activity of ADAMTS-7 and ADAMTS-12 of COMP are associated with COMP degradation in osteoarthritis (OA); (2) whether alpha-2-macroglobulin (a_2M) is a novel substrate for ADAMTS-7 and ADAMTS-12; and (3) whether a_2M inhibits ADAMTS-7 or ADAMTS-12 cleavage of COMP.

Methods: An *in vitro* digestion assay was used to examine the degradation of COMP by ADAMTS-7 and ADAMTS-12 in the cartilage of OA patients; in cartilage explants incubated with tumor necrosis factor-alpha (TNF-α) or interleukin-1-beta (IL-1β) with or without blocking antibodies; and in human chondrocytes treated with specific small interfering RNA (siRNA) to knockdown ADAMTS-7 or/and ADAMTS-12. Digestion of a_2M by ADAMTS-7 and ADAMTS-12 *in vitro* and the inhibition of ADAMTS-7 or ADAMTS-12-mediated digestion of COMP by a_2M were also analyzed.

Results: The molecular mass of the COMP fragments produced by either ADAMTS-7 or ADAMTS-12 were similar to those observed in OA patients. Specific blocking antibodies against ADAMTS-7 and ADAMTS-12 dramatically inhibited TNF-α- or IL-1β-induced COMP degradation in the cultured cartilage explants. The suppression of ADAMTS-7 or ADAMTS-12 expression by siRNA silencing in the human chondrocytes also prevented TNF-α- or IL-1β-induced COMP degradation. Both ADAMTS-7 and ADAMTS-12 were able to cleave a_2M, giving rise to 180- and 105-kDa cleavage products, respectively. Furthermore, a_2M inhibited both ADAMTS-7- and ADAMTS-12-mediated COMP degradation in a concentration (or dose)-dependent manner.

Conclusion: Our observations demonstrate the importance of COMP degradation by ADAMTS-7 and ADAMTS-12 *in vivo*. Furthermore, a_2M is a novel substrate for ADAMTS-7 and ADAMTS-12. More significantly, a_2M represents the first endogenous inhibitor of ADAMTS-7 and ADAMTS-12.

Key words: ADAMTS-7, ADAMTS-12, COMP, Alpha-2-macroglobulin.

Abbreviations: COMP cartilage oligomeric matrix protein, ADAMTS a disintegrin and metalloproteinase with thrombospondin motifs, MMPs matrix metalloproteinases, PCR polymerase chain reaction, TSP thrombospondin, TNF-α tumor necrosis factor-alpha, IL-1β interleukin-1-beta, GAPDH glyceraldehyde-3-phosphate dehydrogenase, SDS-PAGE sodium dodecyl sulfate polyacrylamide gel electrophoresis, OA osteoarthritis, RA rheumatoid arthritis, a_2M a_2-Macroglobulin.

Introduction

Cartilage consists mainly of extracellular matrix (ECM) with very few cells, mostly chondrocytes. Arthritis is characterized by the breakdown of the ECM and subsequent loss of articular cartilage typically mediated by an excessive amount of active proteolytic activity[1]. The ECM is a network of proteins and macromolecules that provides both strength and nutrients for the cells. Articular cartilage is composed of 60–85% water, 15–22% type II collagen, 4–7% aggrecan and less than 5% other matrix proteins such as cartilage oligomeric matrix protein (COMP), decorin and collagens I, V, VI, IX, and XI among others. COMP, a prominent non-collagenous component of cartilage, accounts for approximately 1% of the wet weight of articular tissue[2,3]. COMP is

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a 524-kDa pentameric, disulfide-bonded, multidomain glycoprotein composed of approximately equal subunits (~110 kDa each)[4,5]. COMP fragments have been detected in the cartilage, synovial fluid, and serum of patients with knee injuries, osteoarthritis (OA) and rheumatoid arthritis (RA)[6–8]. In previous studies to identify the physiological enzymes responsible for COMP degradation, we performed a functional genetic screen, which led to the isolation of a disintegrin and metalloprotease with thrombospondin motifs (ADAMTS)-7 and ADAMTS-12 as COMP-binding partners[9,10]. Subsequent studies showed that both ADAMTS-7 and ADAMTS-12 were able to digest COMP *in vitro* and that their levels were significantly upregulated in arthritic cartilage and synovium compared to a normal controls[6–10].

ADAMTS-7 and ADAMTS-12 belong to the metalloproteinase ADAMTS family. The ADAMTS family consists of secreted zinc metalloproteinases with a precisely ordered modular organization that includes at least one thrombospondin (TSP) type I repeat[11]. So far, 19 members have been cloned in this family and some of them have known functions that have been implicated in specific diseases[12]. For instance, ADAMTS-13 mutants have a role in thrombotic thrombocytopenic purpura, a disease characterized by a decrease in the amount of circulating platelets[13]. Mutations in the *ADAMTS-2* gene (procollagen I N-proteinase) cause Ehlers-Danlos syndrome Type VII C, a genetic condition characterized by defects in collagen synthesis, as well as bovine dermatopraxis[14]. A number of ADAMTS members have been implicated in the breakdown of cartilage in OA and RA, including ADAMTS-4 (aggrecanase 1), ADAMTS-5 (aggrecanase 2)[15–18], ADAMTS-7[9] and ADAMTS-12[10].

a_2-Macroglobulin (a_2M) is a member of the α-macroglobulin family of proteins found in the circulation of a broad range of species[19]. Human a_2M is found at relatively high levels (2–4 mg/ml) in plasma and is a tetramer of four identical 185-kDa subunits, each of which has an exposed 39-amino acid ''bait region'' that contains cleavage sites for a variety of proteinases[20,21]. The function of the bait region is to trap the proteinase, potentially accounting for its capacity to bind and inhibit ADAMTS-4, ADAMTS-5 and ADAMTS-10[22,23]. ADAMTS-1 forms a stable complex with a_2M that is dependent on the zinc binding catalytic domain of ADAMTS-1[24].

Inhibition of degradative enzymes can slow or block disease progression. The isolation of physiological inhibitors for the cartilage degradative enzymes is, therefore, of great interest from both a pathophysiological and a therapeutic standpoint. a_2M is an inhibitor of several metalloproteases, including collagenase, stromelysis[25], ADAMTS-4 and ADAMTS-5[23]. In addition, a_2M also associates with ADAMTS-7[26]. The purpose of the study was to examine the potential association of the ADAMTS-7 and ADAMTS-12-mediated cleavage of COMP with OA damage, and the possible role of a_2M as a substrate for and inhibitor of ADAMTS-7 or ADAMTS-12 enzyme activity against COMP.

Materials and methods

SOURCES OF TISSUES

Normal adult articular cartilage was obtained from the knees of patients (mean age 56.7 years, range 43–64 years) who had died of diseases unrelated to arthritis (specimens obtained en bloc from the Musculoskeletal Transplant Foundation). The grade of OA was determined using the Kellgren–Lawrence Grading System[27]. Normal cartilage samples were without radiographic or intra-articular evidence of arthritic disease (Kellgren–Lawrence Grade 0). Arthritic cartilages were obtained (with IRB#: 12758) from patients undergoing elective total knee arthroplasty for end-stage OA with Kellgren–Lawrence Grade of 3 or 4 from the distal femora of eight patients (mean age 58.4 years, range 49–66 years). The samples were then stored at −80°C until analysis.

IN VITRO DIGESTION ASSAY OF COMP BY ADAMTS-7 AND ADAMTS-12

An *in vitro* digestion protocol described previously[9,10] was followed to determine whether the fragments resulted from COMP digestion by ADAMTS-7 and ADAMTS-12 are the same as those seen in the cartilage of OA patients. Briefly, purified COMP was incubated with either recombinant ADAMTS-7 or ADAMTS-12 in a digestion buffer (50-mM Tris–HCl, 100-mM NaCl, 5-mM $CaCl_2$, 2-mM $ZnCl_2$, pH 7.5) for 8 h at 37°C. The digested products were resolved by 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), under reduced condition, and the gel was stained with Coomassie brilliant blue G-colloidal solution.

CARTILAGE EXPLANT CULTURES

Human cartilage was cultured as described previously[28,29] with modifications. Briefly, human knee cartilage was dissected into pieces of diameter of approximately 4 mm by punches of 1- to 2-mm thickness. The cartilage was dispensed into tissue-culture flasks (0.7 g/flask) and incubated overnight in control, serum-free medium Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) containing 25-mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2-mM glutamine, 100-μg/ml streptomycin, 100-IU/ml penicillin, 2.5-μg/ml gentamicin, and 40-units/ml nystatin. Fresh control medium (10 ml) with tumor necrosis factor-alpha (TNF-α, 5 ng/ml) or interleukin-1-beta (IL-1β, 5 ng/ml) (in triplicate for statistical analysis) was then added (day 0). At day 2, the supernatants were harvested for COMP degradation analysis by Western blotting and the cartilage samples were extracted for RNA, as described below. In some cultures, antibodies against ADAMTS-7 or/and ADAMTS-12 (5 μg/ml of anti-ADAMTS-7 or/and ADAMTS-12 rabbit polyclonal antibodies) were added[9,10]. At day 7, culture supernatants were harvested and COMP degradative fragments in the culture supernatants were determined using Western blotting assay.

COMP DEGRADATION ANALYSES IN THE CARTILAGE OF OA PATIENTS

Extracts of normal and OA cartilages and supernatants from cultured cartilage explants were analyzed by Western blotting as previously described[9,10]. Briefly, the samples were loaded on 8% gels and separated by SDS-PAGE under reducing conditions. Separated proteins were transferred to polyvinylidene difluoride membranes and probed with a 1:2500 dilution of rabbit polyclonal anti-COMP antiserum[6,9,30,31]. Subsequently, membranes were incubated with a 1:20,000 dilution of goat anti-rabbit IgG horseradish peroxidase conjugate as the secondary antibody, and the signal detected using the ECL chemiluminescent system (Amersham Pharmacia Biotech, Upsala, Sweden).

ANALYSIS OF ADAMTS-7 AND ADAMTS-12, mRNA IN CULTURED CARTILAGE EXPLANTS

Total RNA was extracted as described previously[9,10] and real-time polymerase chain reaction (PCR) was performed using a sequence-specific probe and primers for ADAMTS-7 (fluorescence-labeled oligonucleotide probes [using 6-carboxy-fluorescein (FAM)] probe: 5′-*AAGCGCTT CCGCCTCTGCAACC*-3′; primers: 5′-*CAGCCTACGCCCAAATACAAA*-3′ and 5′-*CCCTTGTAGAGCATAGCGTCAAA*-3′) and ADAMTS-12 (fluorescence-labeled oligonucleotide probes [using FAM] probe: 5′-AGGA CATCTGTGCTGGTTTCAATCGCC-3′; primers: 5′-CACGACGTGGCTG TCCTTCT-3′ and 5′-CCGAATCTTCATTGATGTTACAACTG-3′). The PCR products obtained was confirmed by direct sequencing of the amplicons. A standard curve with copy numbers ranging from 10^3 to 10^9 was produced using human cartilage cDNA as the template. An *XY* scatter plot was produced using Microsoft Excel software, and the equation $y = mx + b$ (where m = the slope of the standard curve and b = the y intercept of that line) was calculated and R^2 values obtained. As an internal control, 18S rRNA was analyzed in parallel by using the Endogenous Control Human rRNA kit (Applied Biosystems, Foster City, CA).

PCR reactions for all samples were performed in duplicate in 96-well optical plates with 5 ng of cDNA (1 ng of cDNA for the 18S rRNA), 100-nM probe, 200-nM each primer, and 10.0 μl of TaqMan Universal 2× PCR Master Mix (PE-Applied Biosystems, St. Louis, MO) in a 20-μl reaction volume. The amplification reaction was carried out over 40 cycles (an initial holding stage of 2 min at 50°C and then 10 min at 95°C, followed by a two-step cycling program of 15 s at 95°C and 1 min at 60°C).

KNOCKDOWN OF ADAMTS-7 AND ADAMTS-12 BY SPECIFIC SMALL INTERFERING RNA (siRNA)

The human chondrocyte cell line, C-28/12, was used as a model for analyzing the efficiency of knockdown by the siRNAs and for determining the consequences of knockdown of ADAMTS-7 and ADAMTS-12 on COMP degradation. The cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS). Two regions of human ADAMTS-7 or/and ADAMTS-12 were targeted for siRNA using mammalian expression pSUPER vector (OligoEngine, Seattle, WA) according to the manufacturer's instructions. To generate each siRNA, equimolar amounts of complementary sense and antisense strands were mixed and annealed slowly by cooling to 10°C in a 50-μl reaction buffer (100-mM NaCl and 50-mM HEPES pH 7.4). The annealed oligos were inserted into the BglII/HindIII sites of pSUPER vector. The resulting plasmids and control vector pSUPER were co-transfected with the corresponding expression plasmid into C28I2 cells using LipofectAMINE 2000 reagent (Invitrogen, Rockville, MD) and the levels of ADAMTS-7 or/and ADAMTS-12 were monitored using immumofluorescence cell staining as described below. The data demonstrated that the siRNA 5′-ACCTAAAGATCACGCACCA-3′ and the siRNA 5′-ACACATCACACACACCCAA-3′ were able to efficiently reduce the expression of human ADAMTS-7 and ADAMTS-12, respectively. The C-28/12 cells were then transfected with the siRNA described above [i.e., ADAMTS-7 siRNA (siTS-7), ADAMTS-12 siRNA (siTS-12), both (siTS-7 + siTS-12) or pSUPER control (CTR)] and cultured in the presence of TNF-α (5 ng/ml) or IL-1β (5 ng/ml). After incubation for 7 days, the media were collected and assayed by Western blotting with anti-COMP antibody.

IMMUMOFLUORESCENCE CELL STAINING

Briefly, cultures plated on chamber slides (Nalge Nunc International, Naperville, IL) were fixed with cold 100% methanol and air-dried. After rehydration in phosphate buffered saline (PBS) and blocking with 30% goat serum for 30 min, the cells were incubated with primary antibodies against ADAMTS-7 (Santa Cruz; diluted 1:100) or ADAMTS-12 (diluted 1:100) for 1 h. Secondary antibodies against rabbit IgG conjugated with FITC (Santa Cruz; diluted 1:100) were applied for 45 min, followed by an incubation with 0.5 mg of 49,69-diamidino-2-phenylindole dihydrochloride (DAPI) for 5 min. The specimens were observed under a fluorescence microscope with appropriate optical filters. Microscopic images were captured using the Image program (Media Cybernetics, Silver Spring, MD) and an Olympus microscope.

DIGESTION ASSAY OF a_2M by ADAMTS-7 AND ADAMTS-12 *IN VITRO*

To determine whether ADAMTS-7 and ADAMTS-12 cleave a_2M, increasing amounts of recombinant ADAMTS-7 and ADAMTS-12[9,10] were incubated overnight at 37°C with 140- or 200-nM human a_2M (Sigma-Aldrich, St. Louis, MO) in 50-mM Tris–HCl, pH 7.5, 100-mM NaCl, and 10-mM $CaCl_2$. Subsequently, reaction products were analyzed by 8% SDS-PAGE under non-reducing conditions and the gel was stained with Coomassie brilliant blue R-250.

INHIBITION ASSAYS OF a_2M ON ADAMTS-7 OR ADAMTS-12 DIGESTION OF COMP

To test the ability of a_2M to inhibit ADAMTS-7 and ADAMTS-12 cleavage of COMP, recombinant ADAMTS-7 or ADAMTS-12 was preincubated with various concentrations of a_2M for 2 h at 37°C. Then purified COMP was added into the above mixture in the digestion buffer (50-mM Tris–HCl, 100-mM NaCl, 5-mM $CaCl_2$, 2-mM $ZnCl_2$, pH 7.5) for two more hours at 37°C. The digested products were resolved by 8% non-reduced SDS-PAGE gel, and the gel was either stained with Coomassie brilliant blue G-colloidal solution or detected using Western blotting with anti-COMP antibody[9,10,32].

STATISTICAL TEST

Two-sample Student's *t* test was used to determine significant differences ($P < 0.05$) of the levels of ADAMTS-7 and ADAMTS-12 between control and TNF-α- or IL-1β-treated cartilage explants.

Results

THE SIZES OF THE COMP FRAGMENTS PRODUCED BY ADAMTS-7 OR ADAMTS-12 ENZYME ACTIVITY ARE SIMILAR TO THOSE IN CARTILAGE FROM OA PATIENTS

To elucidate the importance of ADAMTS-7- or ADAMTS-12-mediated COMP degradation *in vivo*, we determined whether OA cartilage contained the same fragment as we saw in ADAMTS-7- or ADAMTS-12-mediated COMP digestion *in vitro*. For this purpose, we analyzed the cartilage from six OA patients and COMP fragments produced by *in vitro* COMP digestion with the recombinant ADAMTS-7 or ADAMTS-12 using Western blotting with anti-COMP antibodies (Fig. 1). An approximately 110-kDa fragment (arrow 2) that was produced by digestion with ADAMTS-12 (lane 1) and ADAMTS-7 (lane 2) was an abundant component of all OA cartilage samples (lanes 3–8); intact COMP monomer was also detected (arrow 1). Interestingly, an additional fragment (arrow 3) was observed in OA samples that was absent in the *in vitro* COMP digestion assay with ADAMTS-12 and ADAMTS-7, suggesting that additional enzyme(s) may contribute to COMP degradation in OA patients. Note that only intact COMP was detected in the normal cartilage (lane 9).

INDUCED EXPRESSION OF ADAMTS-7 AND ADAMTS-12 BY TNF-α AND IL-1β

We next investigated whether TNF-α and IL-1β, two major inflammatory cytokines that induce the expression of a number of metalloproteinases involved in the development and progression of arthritic diseases[33–35], could regulate the expression of ADAMTS-7 and ADAMTS-12. Human cartilage explants were cultured in the absence or presence of either 5 ng/ml of TNF-α or 5 ng/ml of IL-1β for 1 day in serum-free medium and real-time PCR was performed [see Fig. 2(A)]. Both TNF-α and IL-1β induced the mRNA expression of ADAMTS-7 and ADAMTS-12 compared to untreated cartilage explants.

ANTIBODIES AGAINST ADAMTS-7 AND ADAMTS-12 ANTIBODY DRAMATICALLY INHIBITS TNF-α- OR IL-1β-INDUCED COMP DEGRADATION

Since both TNF-α and IL-1β upregulate ADAMTS-7 and ADAMTS-12, two enzymes known to degrade COMP, we next determined whether these enzymes could account specifically for the COMP degradation induced by TNF-α or IL-1β in the cartilage organ culture system. Since TNF-α and IL-1β are known to induce the expression of various

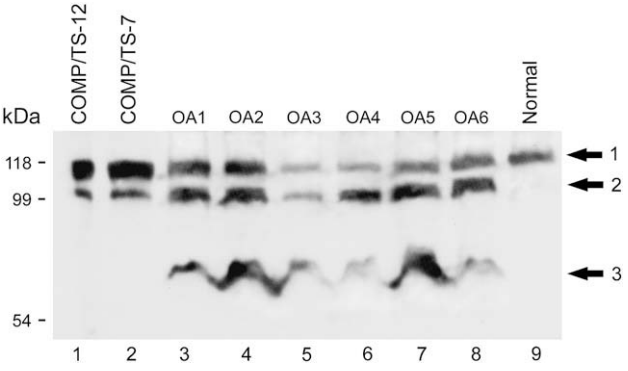

Fig. 1. Western blotting analysis of human OA cartilage samples and ADAMTS-7 (TS-7)- and ADAMTS-12 (TS-12)-mediated COMP digestion. Samples were resolved on 8% SDS-PAGE gels, under reducing conditions, and COMP was detected using an anti-COMP antiserum. Intact COMP monomer, 110-kDa fragment and additional fragment in OA cartilage are indicated with arrows 1, 2, 3, respectively.

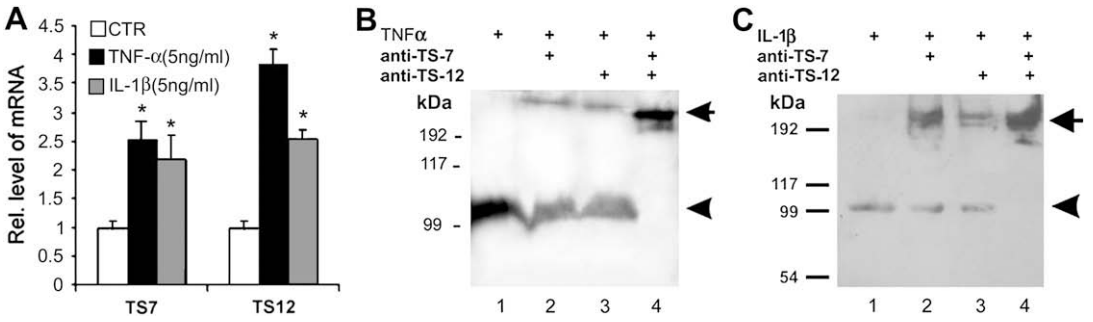

Fig. 2. ADAMTS-12 and ADAMTS-7 blocking antibodies inhibit TNF-α- or IL-1β-induced COMP degradation. (A) Upregulation of ADAMTS-7 and ADAMTS-12 by TNF-α and IL-1β. The units are arbitrary and the leftmost bar in each group indicates a relative level of 1. $^*P < 0.05$ vs untreated controls. (B and C) Antibody blocking assays. OA cartilage explants were cultured in the presence of 5 ng/ml of TNF-α (B) or 5 ng/ml of IL-1β (C) with blocking antibodies, as indicated, for 7 days. The media were separated on non-reduced SDS-PAGE gels and COMP was detected using an anti-COMP antibody. Intact COMP and its degradative fragment are indicated with arrow and arrowhead, respectively.

metalloproteinases, including ADAMTS-4[34,36], COMP degradation might be due to other enzymes alone or in combination with ADAMTS-7/ADAMTS-12 rather than to ADAMTS-7/ADAMTS-12 alone. To determine whether ADAMTS-7 or/and ADAMTS-12 is directly involved in the COMP degradation induced by these two proinflammatory cytokines, we compared COMP degradation in the absence or presence of ADAMTS-12 and/or ADAMTS-7 blocking antibody[9] [Fig. 2(B) and (C)]. TNF-α and IL-1β treatments resulted in a 110-kDa COMP fragment (lane 1, indicated by the arrowhead). This fragment was reduced in the presence of either ADAMTS-12 (lane 2) or ADAMTS-7 (lane 3) blocking antibody; in addition, COMP degradation was totally blocked by a combination of these two antibodies and intact COMP was observed (lane 4, indicated by the arrow), clearly indicating that ADAMTS-12 and ADAMTS-7 are important in TNF-α- and IL-1β-induced COMP degradation. Note that control antibody did not show any blocking activity (not shown).

INHIBITION OF ADAMTS-7 OR/AND ADAMTS-12 EXPRESSION *VIA* siRNA-MEDIATED SILENCING PREVENTS COMP DEGRADATION IN HUMAN CHONDROCYTES

To further verify the importance of ADAMTS-7 and ADAMTS-12 in degrading COMP *in vivo*, we first suppressed ADAMTS-7 or/and ADAMTS-12 gene expression in human chondrocytes using the siRNA approach. We identified 19-nucleotide gene-specific sequences for ADAMTS-7 and ADAMTS-12, respectively, and then generated pSUPER−siTS-7 and pSUPER−siTS-12 constructs encoding siRNAs targeting the specific gene sequences. Immunofluorescent cell staining with human C-28/I2 chondrocytes transfected with either pSUPER−siTS-7, pSUPER−siTS-12 or pSUPER vector demonstrated that expression of the specific siRNAs efficiently reduced the levels of the corresponding proteins [Fig. 3(A)]. Next we examined whether the siRNA knockdown of ADAMTS-7 or/and ADAMTS-12 would affect COMP degradation. The

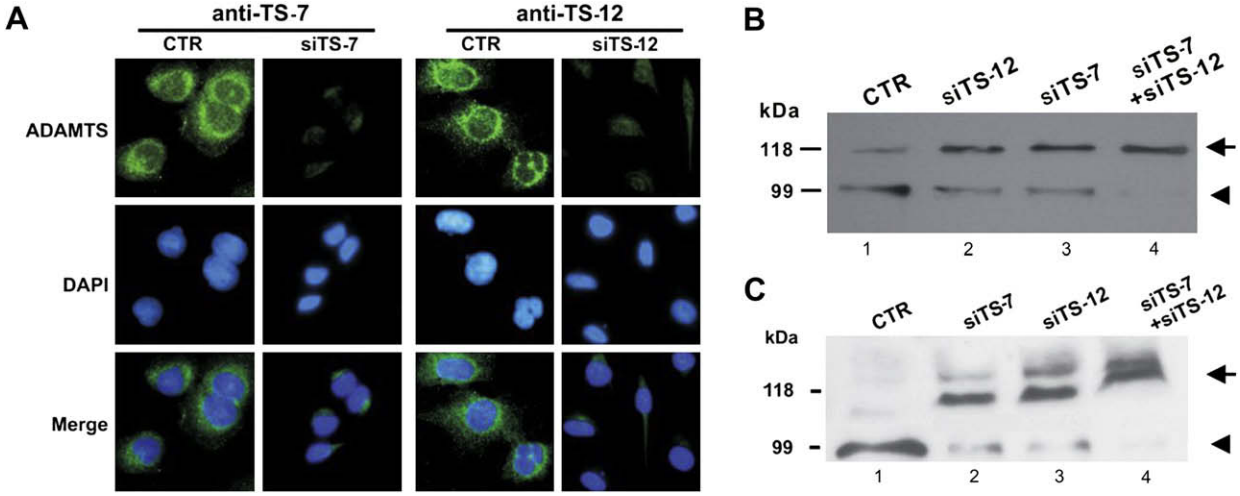

Fig. 3. Reduced expression of ADAMTS-7 or/and ADAMTS-12 by siRNA silencing inhibits the degradation of COMP in human chondrocytes. (A) siRNAs against ADAMTS-7 and ADAMTS-12 efficiently suppress the expression of their target molecules, assayed by immumofluorescence cell staining. Immortalized human chondrocytes, C-28/I2, transfected with either pSUPER plasmid (CTR), pSUPER−ADAMTS-7 siRNA (siTS-7) or pSUPER−ADAMTS-12 siRNA (siTS-12) were stained with either anti-ADAMTS-7 (left panel) or anti-ADAMTS-12 (right panel). The nuclei were stained with DAPI and the overlapping of these two signals is shown as "merge". (B and C) Knockdown of either ADAMTS-7 or/and ADAMTS-12 dramatically inhibits COMP degradation. The C-28/12 cells were transfected with either ADAMTS-7 siRNA (siTS-7), ADAMTS-12 siRNA (siTS-7), both (siTS-7 + siTS-12) or pSUPER control (CTR), were cultured in the presence of 5 ng/ml of TNF-α (B) or 5 ng/ml of Il-1β (C) for 7 days. The media were separated on reduced SDS-PAGE gels and COMP was detected using an anti-COMP antibody. Intact COMP and its degradative fragment are indicated with arrow and arrowhead, respectively.

C-28/I2 chondrocytes were transfected with either pSUPER−siTS-7, pSUPER−siTS-12 or both and cultured with serum-free medium containing TNF-α or IL-1β for 1 week. Western blotting with anti-COMP antibody [Fig. 3(B) and (C)] showed a robust COMP degradative fragment in the medium from TNF-α- or IL-1β-treated cultures (lane 1). However, the intensity of the COMP fragment was reduced in the media collected from the cells transfected with pSUPER−siTS-7 or pSUPER−siTS-12 (lanes 2 and 3). Especially, the degradative fragment was barely detectable when the cells were co-transfected with pSUPER−siTS-7 and pSUPER−siTS-12 (lane 4). Collectively, these results further indicated that both ADAMTS-7 and ADAMTS-12 were critical for the TNF-α- or IL-1β-induced COMP degradation.

a_2M IS A NOVEL SUBSTRATE FOR ADAMTS-7 AND ADAMTS-12

Since a_2M associates with ADAMTS-7[26], and a_2M inhibits ADAMTS-4 and ADAMTS-5 by competitive inhibition upon cleavage activity by the bait region of ADAMTS-4/-5[23], we investigated whether a_2M is a also a substrate for ADAMTS-7 and ADAMTS-12. We first examined the digestion of a_2M by ADAMTS-7 by incubation of 140 nM of a_2M with various concentrations of purified recombinant ADAMTS-7 and resolved the digests on Coomassie blue-stained SDS-PAGE. Intact a_2M in its tetramer form was detected at a molecular mass of ~700 kDa [Fig. 4(A), lane 1]. One major a_2M cleavage product with the apparent molecular weight of approximately 180 kDa was observed when 10 nM of ADAMTS-7 was applied and the intensity of this band strengthened gradually with increasing concentrations of ADAMTS-7 [Fig. 4(A)]; a faint degradative fragment with the molecular weight of 105 kDa was observed using ADAMTS-7 at 430 nM or higher [Fig. 4(B)]. A similar cleavage pattern of a_2M was observed using recombinant ADAMTS-12 [Fig. 4(C)].

a_2M INHIBITS ADAMTS-7- AND ADAMTS-12-MEDIATED COMP DEGRADATION

Since a_2M can be digested by ADAMTS-4 and ADAMTS-5 and inhibits the cleavage of aggrecan by these enzymes[23], we next examined whether a_2M, as a substrate of ADAMTS-7 and ADAMTS-12, also acts as an competitive inhibitor of the degradation of COMP. ADAMTS-7 or ADAMTS-12 at a concentration of 330 nM were preincubated with various amounts of a_2M for 2 h at 37°C. After the preincubation COMP was added to a final concentration of 170 nM, and then the reactions were carried out for another 2 h at 37°C. The products were first analyzed on a non-reduced SDS-PAGE gel and visualized by Coomassie blue staining [Fig. 5(A) and Fig. 6(A)]. Accompanying

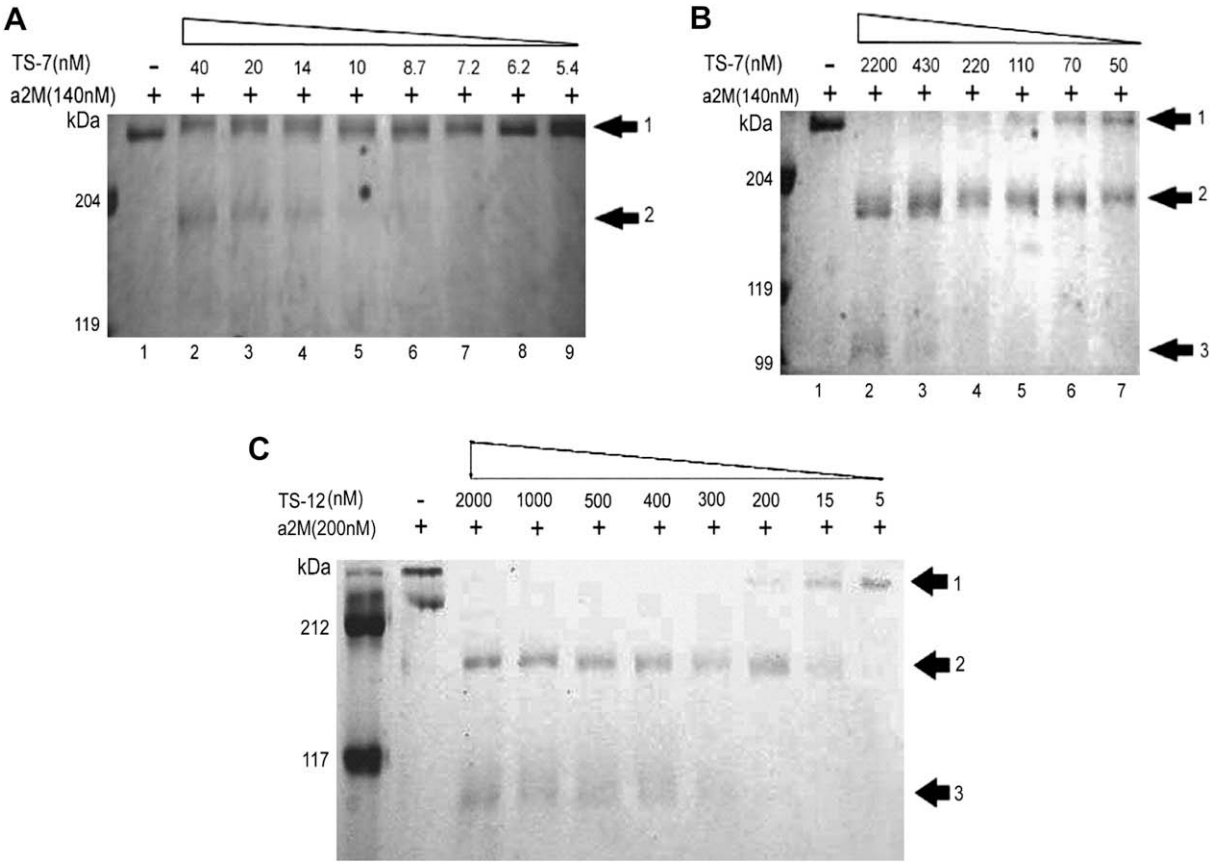

Fig. 4. Cleavage of a_2M by ADAMTS-7 and ADAMTS-12. *In vitro* digestion assay of a_2M by lower (A) or higher (B) amount of ADAMTS-7. 0.14-μM a_2M was incubated in the absence or presence of various amount of ADAMTS-7, as indicated, for 2 h at 37°C. The products were then separated by 8% non-reduced SDS-PAGE and visualized by Coomassie blue staining. (C) Digestion of a_2M by ADAMTS-12. 0.10 μM a_2M was incubated in the absence or presence of various amount of ADAMTS-12, as indicated, for 2 h at 37°C. The products were then separated by 6% non-reduced SDS-PAGE and visualized by Coomassie blue staining. Arrows 1, 2, 3 indicate the intact a_2M, ~180 kDa and 105 kDa resulted fragments, respectively.

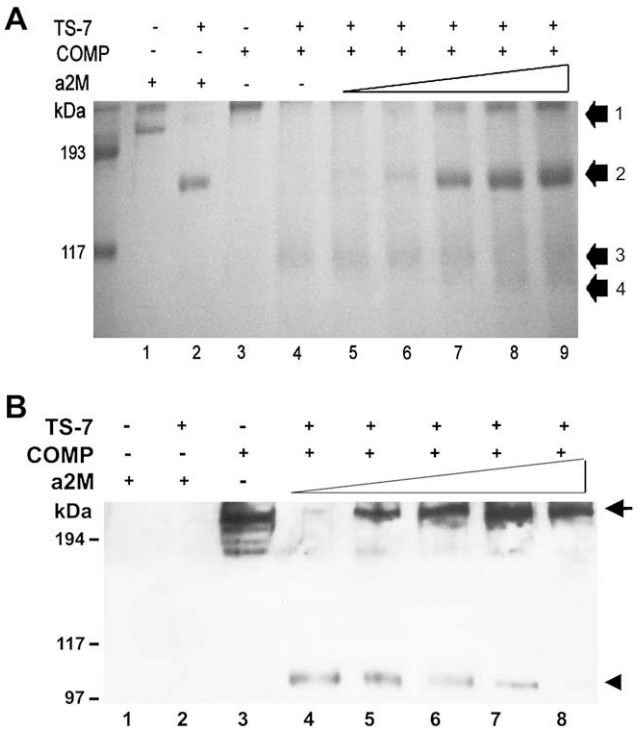

Fig. 5. a_2M inhibits ADAMTS-7-mediated COMP degradation in a dose-dependent manner, assayed by Coomassie blue staining (A) and Western blotting (B). 0.33-μM ADAMTS-7 was first incubated with increasing concentrations of a_2M, as indicated, for 2 h at 37°C, then 0.17-μM COMP was added and allowed to incubate for additional 2 h at 37°C. The resulting products were analyzed by 8% non-reduced SDS-PAGE and visualized by either Coomassie blue staining (A) or Western blotting with anti-COMP antibody (B). Arrows 1, 2, 3 and 4 in (A) indicate the intact COMP, the 180-kDa fragment of a_2M, the 110-kDa fragment of COMP and the 105-kDa fragment of a_2M, respectively. In panel (B), arrow and arrowhead indicate intact COMP and its degradative fragment, respectively. Lanes 1 and 2 indicate that anti-COMP antibody does not cross-recognize neither intact a_2M nor its 180-kDa degradative fragment. Lanes 4–8 indicate that intact COMP was protected from ADAMTS-7 cleavage by a_2M in a dose-dependent manner.

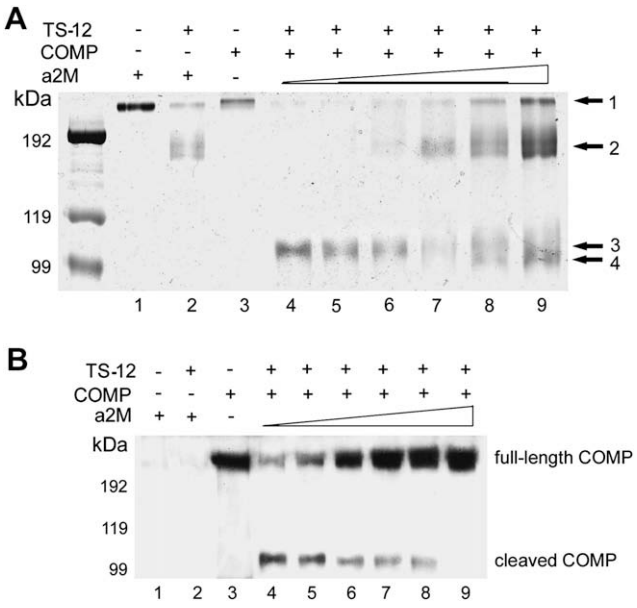

Fig. 6. a_2M inhibits ADAMTS-12-mediated COMP degradation in a does-dependent manner, assayed by Coomassie blue staining (A) and Western blotting (B). 0.20-μM ADAMTS-12 was first incubated with increasing concentrations of a_2M, as indicated, for 2 h at 37°C, then 0.10-μM COMP was added and allowed to incubate for an additional 2 h at 37°C. The resultant products were analyzed by 8% non-reduced SDS-PAGE and visualized by either Coomassie blue staining (A) or Western blotting with anti-COMP antibody (B). Arrows 1, 2, 3 and 4 in panel A indicate the intact COMP, the 180-kDa fragment of a_2M, the 110-kDa fragment of COMP and the 105-kDa fragment of a_2M, respectively. In panel B, the intact COMP and its 110-kDa degradative fragment are indicated. Lanes 1 and 2 indicate that anti-COMP antibody does not cross-react with either intact a_2M or its 180-kDa degradative fragment. Lanes 4–9 indicate that intact COMP was protected from ADAMTS-7 cleavage by a_2M in a dose-dependent manner.

the increase of a_2M, the intensities of the180 kDa (arrow 2) and 105 kDa (arrow 4) fragments of a_2M became stronger, whereas the 110-kDa COMP degradative fragment became weaker and finally not visible (arrow 3) and the intact COMP (arrow 1) band appeared [Fig. 5(A) and Fig. 6(A)]. Since both intact a_2M (700 kDa) and COMP (550 kDa) were retained at the very top of the gel, we next performed Western blotting with anti-COMP antibodies to determine whether the top band (arrow 1) was COMP rather than a_2M [Fig. 5(B) and Fig. 6(B)]. Western blotting with anti-COMP antibodies [these do not cross-react with a_2M and its digested products, lanes 1 and 2 of Fig. 5(B) and Fig. 6(B)] clearly demonstrated that a_2M efficiently protects COMP from degradation by either ADAMTS-7 or ADAMTS-12.

Discussion

ADAMTS family proteins have been implicated in the pathogenesis of different diseases, including arthritis[37–43]. We previously reported that ADAMTS-7 and ADAMTS-12, two members in this family sharing similar domain organization and structure, associated with and cleaved COMP in the *in vitro* digestion system, and their levels were significantly elevated in the cartilage and synovium of patients with arthritis[9,10]. The present study provides insight into the importance of ADAMTS-7 and ADAMTS-12 in the degradation of COMP in the course of arthritis, since the size of the COMP fragment produced by ADAMTS-7 or ADAMTS-12 is similar to that of COMP degradative fragments seen in OA patients (Fig. 1). Antibody blocking assays with cartilage explants have been widely used[44–46]. Using this model we found that anti-ADAMTS-7 and/or anti-ADAMTS-12 antibody dramatically inhibited COMP degradation induced by TNF-α and IL-1β, two key cytokines in the progression of arthritis that induce the expression of ADAMTS-7 and ADAMTS-12 (Figs. 2 and 3). In addition, both anti-ADAMTS-7 and anti-ADAMTS-12 antibodies did not inhibit ADAMTS-4-mediated COMP digestion in an *in vitro* assay (not shown). These data indicated that ADAMTS-7 and ADAMTS-12 are important for the cytokine-induced COMP degradation. The present study also presents evidence that a_2M is a novel substrate for ADAMTS-7 and ADAMTS-12 (Figs. 4 and 5) and acts as an inhibitor of COMP degradation mediated by these two enzymes (Figs. 5 and 6).

Nineteen distinct human ADAMTS gene products have been cloned and can be divided into five subgroups based on their known functions[12]. The first of the divisions,

consisting of ADAMTS-1, ADAMTS-4, ADAMTS-5, ADAMTS-8, ADAMTS-9, ADAMTS-15 and ADAMTS-20, cleave aggrecan[12]. ADAMTS-1 also cleaves the related hyalectan versican at analogous sites and ADAMTS-4 has been demonstrated to cleave brevican[47]. Among them, ADAMTS-4 and ADAMTS-5 have been best characterized and implicated in aggrecan degradation in OA[16]. ADAMTS-5 is probably the major aggrecanase responsible for aggrecan degradation *in vivo*[41,48]. ADAMTS-4 has been also shown to cleave COMP as well as fibromodulin and decorin[49–51]. A second subgroup contains ADAMTS-2, ADAMTS-3 and ADAMTS-14. ADAMTS-2 cleaves the amino peptides of type I, type II and type III procollagens[52,53], ADAMTS-3 has since been identified as a type II procollagen N-propeptidase, whose expression is about fivefold that of ADAMTS-2 in cartilage[54]. ADAMTS-14 has been identified as a homologue of ADAMTS-2, functioning as the major type I procollagen N-propeptidase activity in tendon[55]. ADAMTS-13, a von Willebrand factor-cleaving protease, stands as one subgroup[56,57]. ADAMTS-7 and ADAMTS-12 that specifically associate with and degrade COMP represent another subgroup[9,10,26,58]. And the remaining ADAMTS members form a loosely defined subgroup with unknown functions[12].

In addition to ADAMTS-7 and ADAMTS-12, ADAMTS-4 and several members of the family of matrix metalloproteinases (MMPs), including interstitial collagenase (MMP-1), collagenase-3 (MMP-13), stromelysin-1 (MMP-3), gelatinase-B (MMP-9), MMP-19, and enamelysin (MMP-20) also digest purified COMP *in vitro*. Here we also show that, an additional fragment (Fig. 1, small arrowhead) was observed in OA samples that was absent in ADAMTS-12- and ADAMTS-7-mediated COMP digestion, suggesting that additional enzyme(s) may contribute to COMP degradation in OA patients. In addition to COMP, a recent report revealed that aggrecan was also a substrate of ADAMTS-12[59], suggesting that competitive substrate might lead to the enzyme inhibition. In addition, several reports suggest that COMP may function to stabilize the articular cartilage ECM by specific cation-dependent interactions with matrix components, including collagen types II and IX, fibronectin, aggrecan, and matrilin-1, -3, and -4[32,60–63]. Thus, it is conceivable that the inhibition of COMP degradation may stabilize the cartilage matrix and in turn affect the degradation of the main macromolecules, including collagens and aggrecan.

Full length a_2M is approximately 185 kDa and forms a tetramer. It may be cleaved by ADAMTS-4 and ADAMTS-5, two major aggrecanases, and can inhibit their activities[23]. In this study we demonstrated that a_2M is also a substrate for ADAMTS-7 and ADAMTS-12, two known COMP-associating and -degrading metalloproteinases (Figs. 4 and 5) and prevents the degradation of COMP by these enzymes in a does-dependent manner (Figs. 5 and 6). Degradation of a_2M by ADAMTS-7 and ADAMTS-12 gives rise to two fragments, one major 180-kDa and one minor 105-kDa fragment, suggesting that a_2M may contain two cleavage sites of ADAMTS-7 and ADAMTS-12 (Figs. 4 and 5). Tortorella *et al.* have shown that ADAMTS-4 and ADAMTS-5 digested a_2M at one major sensitive site and at additional insensitive sites with much less efficiency[23].

Evidence showing the importance of ADAMTS-7 and ADAMTS-12 for the *in vivo* degradation of COMP, identification of a_2M as a novel substrate for ADAMTS-7 and ADAMTS-12, and subsequent characterization of its inhibitory activities on the degradation of COMP by ADAMTS-7 and ADAMTS-12 significantly extends our understanding of the degradative events that occur in joint disorders. These findings will also contribute to our ability to monitor the biological and physical properties of cartilage ECM, and provide us with promising therapeutic targets, including a_2M or its derivatives, for treating or preventing ECM degeneration.

Conflict of interest

The authors have no conflict of interest.

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