The alteration of A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) in the knee joints of osteoarthritis mice

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ABSTRACT

The A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family is gradually being recognized as an important family of mediators that, along with the matrix metalloproteinases (MMPs), control the degradation process in osteoarthritis (OA). The objective of this study was to uncover the detailed alterations of ADAMTS1, ADAMTS2, and ADAMTS5 in the knee joint of OA mice. The OA model was established by anterior cruciate ligament transection (ACLT) on the knee joints of C57BL/6 J mice. The mice showed representative phenotypes of ACLT-induced OA, including obvious deterioration of the cartilage, reductions in the collagen and proteoglycan components in the cartilage matrix of OA mice, and increased inflammation and osteoclast activity. By qPCR, the gene expression levels of Adamts1, −2, and −5 were the top-ranked among ADAMTS1-5 in cartilage/chondrocytes, osteogenic tissue/osteoblasts, and cortical bone/osteocytes. Moreover, the protein expression levels of ADAMTS1, −2, and −5 were all increased in articular cartilage, the growth plate, and subchondral bone of the knee joint. The results suggest the important roles of ADAMTS1, −2, and −5 in OA disease, which will be helpful in further research on degenerative changes in OA.

KEYWORDS

ADAMTS; osteoarthritis of mice; anterior cruciate ligament transection; knee joint; articular cartilage; subchondral bone; growth plate

Introduction

Osteoarthritis (OA) is a degenerative joint disease characterized by progressive degeneration and destruction of the articular cartilage, along with alterations in the subchondral bone, meniscus, synovium, and surrounding joint tissues, eventually leading to joint dysfunction or disability[1]. OA mainly occurs in middle-aged and elderly people, who have a high incidence: more than 50% of people over 65 years old develop the disease. Clinical features of OA include joint pain, limited joint movement, joint deformity, and bone friction sensation, which severely decrease the quality of life of OA patients and create an enormous social and economic burden.

It is generally agreed that OA has a multifaceted etiology related to many potential risk factors [2,3], but its etiology and pathogenesis are not clearly understood. Cartilage dysfunction and destruction are critical in the pathogenesis of OA. Chondrocytes, as the only cell type in cartilage, play an important role in the maintenance of cartilage homeostasis by synthesizing and degrading extracellular matrix (ECM), including collagen and proteoglycan [1,4]. The collagen fiber network provides the tensile strength of articular cartilage, and the proteoglycan that fills in the spaces in the network is hydrophilic, to bind water molecules and resist compressive forces [5], giving this molecule a helpful buffering role against the stresses put on articular cartilage. Once chondrocytes are exposed to the inflammatory environment of OA, anabolism is inhibited and catabolism is promoted, which accelerate the breakdown of cartilage. Proteoglycans are degraded by certain enzymes, and then the collagen fibrous network is destroyed, leading to irreversible progression of articular cartilage damage [3,5]. Aggrecan, the major component of proteoglycan, can be an early sign of cartilage destruction in osteoarthritis.

A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) is a family of 19 secreted proteinases that take part in several physiological and pathological processes of tissues by performing multiple functions. According to structure and function, the ADAMTS family is divided into four classifications: (1) aggrecanases/proteoglycanases (ADAMTS1, −4, −5, −8, −9, −15, and −20); (2) procollagen N-propeptidases
(ADAMTS2, −3 and −14). (3) cartilage oligomeric matrix proteinases (ADAMTS7 and −12); and (4) the von Willebrand factor proteinase (ADAMTS13). There is also a fifth class, unknown, or orphan enzymes (ADAMTS6, −10, −16, −17, −18, and −19) [6]. As secreted proteases, ADAMTSs are closely related to the degradation of the extracellular matrix and contributes to osteoarthritis development by cleaving ECM [7,8].

Although the ADAMTS family is gradually being recognized as important mediators that, along with the matrix metalloproteinase (MMP) family, control the degradation process in OA, a detailed investigation focused on the ADAMTS family in the whole pathological joint has not been done. The purpose of this study was to investigate the expression of the ADAMTS family in the knee joint of OA mice.

Methods

OA model mice induced by anterior cruciate ligament transection

Four-week-old mice (C57BL/6 J) were obtained from Dashuo Biological Institute (Chengdu, China) and all experimental procedures were approved and conducted by the Institutional Animal Care and Use Committees of Sichuan University (approval number SYXX(Chuan) 2018–185). The present study followed international, national, and/or institutional guidelines for humane animal treatment and complied with relevant legislation. Anterior cruciate ligament transection (ACLT) surgery was used to establish the OA model. A total of 20 male mice were randomly divided into the Sham group (n = 10) and ACLT group (n = 10). In the Sham group, the joint cavity was opened at the left knee joint without cutting the anterior cruciate ligament, while ACLT surgery was performed on the right knee joint in the ACLT group. The surgery was performed as follows. Anesthesia was induced by intraperitoneal injection using 250 mg/kg tribromoethanol (T48402, Sigma-Aldrich, USA). Lateral arthrotomy was performed on the right leg, the patella was medially reclinied, and the knee was fully flexed to visualize the ACL. The ACL was transected without affecting the surrounding tissues. After surgery, the mice were cared and had no obvious abnormal behavior, including death, or changes of weight and diet. The mice were euthanized by CO2 inhalation at 4 weeks after surgery, and the hind legs were dissected and fixed in 4% paraformaldehyde (BL539A, Biosharp, China) for 24 h at room temperature (RT) and then stored in 75% ethyl alcohol. For qPCR, the samples of articular cartilage were taken by scraping the surface of joint with a scalpel. Subchondral bone was exposed and osteogenic tissue samples were collected. The cortical bone samples were collected from the middle shaft of femurs without bone marrow. The chondrocytes were cultured from cartilage tissues of new-born C57BL/6 J mice. The osteoblasts were cultured from skull bone of new-born C57BL/6 J mice. The osteocytes were cell line MLO-Y4 (Cat. No. 01, JENNIO Biological Technology, Guangzhou, China, http://gzjno.foodmate.net/sell/itemid-711300.shtml).

Micro-computed tomography

Micro-computed tomography (μCT 50, Scanco Medical, Switzerland) was used to observe and evaluate the knee joint morphology of mice. Samples were scanned at 10 μm voxel size and then analyzed using the evaluation software provided by the manufacturer. The region of interest (ROI) was the subchondral trabecular bone in tibia, and five serial layers were selected to analyze the ratio of bone volume to tissue volume.

Hematoxylin and Eosin Staining

The acquired tissues were decalcified in 10% aq. EDTA (MW 229.24 Technical grade, 05 001 0882, Chron Chemicals, China) for 8 days. The pH of the EDTA solution was adjusted to 7.4 ± 0.1 with hydrochloric acid (HCl) (06 037 03345, Chron Chemicals). The tissues were processed through ascending ethanol concentrations 30 min per change cleared in xylene and infiltrated with heated paraffin for 60 min for three changes. Paraffin-embedded tissues were sectioned at 8 μm with a rotary microtome. The sections were deparaffinized in xylene for 15 min, immersed in descending gradient ethanol for 3 min per concentration, and hydrated in double-distilled water (ddH2O) for 10 min. The procedure above was repeated at the beginning of all staining methods. Hematoxylin and Eosin Staining Kit (O105OS-1/C0105S-2, Beyotime Biotechnology, China) was used to stain sections: hematoxylin for 5 min, rinsed with water, differentiated with 1% acid (HCl) alcohol, blued in ammonia water (1:500 dilution) ammonia hydroxide (AX1308, Sigma Aldrich), rinsed in water and stained in Eosin for 1 min. After dehydrating in ascending gradient of ethanol, the
sections were cleared in xylene for 5 min, a coverglass mounted in neutral balsam (G8590, Solarbio, China). All H&E and following stained sections were examined a light microscope (BX53, Olympus, Japan).

**Safranin O/Fast green staining**

The hydrated tissue sections were stained with Weigert’s iron hematoxylin for 5 min and differentiated in 1% acid alcohol for 10 sec. Following a protocol according to Zhang et al. [9], 0.02% acq. fast green was applied to stain the background for 2 min. After a brief ddH2O rinse, sections were placed in 0.1% safranin O solution (HT90432, Sigma-Aldrich) for 15 min. Sections were dehydrated, cleared, and coverglass mounted as described for H&E.

**Masson Trichrome staining**

Masson trichrome stain, commonly used to detect collagen fibers and muscle fibers, was done using Massons Trichrome Stain Kit and instructions (G1340, Solarbio). The sections were dehydrated, cleared, and mounted in neutral balsam.

**Tartrate-resistant acid phosphatase staining**

Tartrate-resistant acid phosphatase (TRAP) staining, for identifying osteoclasts, was done using the protocol for Acid Phosphatase (Leukocyte (TRAP) kit) (387A, Sigma-Aldrich). The hydrated tissue sections were stained with TRAP solution for 1 h at RT and rinsed in ddH2O for 5 min. Alcian blue (Alcian Blue Stain Kit, pH 2.5, G1560, Solarbio) was applied to stain the background for 5 min. Stained sections were dehydrated, cleared, mounted in neutral balsam.

**ELISA**

The whole blood samples were obtained from the eye retro-orbital sinus when mice were euthanized. Blood samples were centrifuged (5000 rpm, 10 min), and the serum fraction was collected for enzyme-linked immunosorbert assay (ELISA). The concentrations of TNF-α and IL-1β were measured by using ELISA kits (Mouse TNF-α PT512 and Mouse IL-1β PI301, Beyotime Biotechnology) according to the manufacturer protocols. The dilution ratio was consistent with the positive control provided in kit. The optical density at 450 nm was measured with a microplate reader (TECAN, Switzerland).

| Table 1. Primer pairs in this study. |
|-------------------------------------|
| mRNA                              | Primer pairs                                |
| GAPDH (NM_001289726.1)            | Forward: AGGTGTCTCCTGGCGACTTCA              |
|                                    | Reverse: CAAGAAATAGCGTGAACAAA               |
| ADAMTS1 (NM_001002005.2)          | Forward: GGGAAGCTGCTCAAGAAACC              |
|                                    | Reverse: ACCGTGAGCGACTGTTTG                |
| ADAMTS2 (NM_001002000.5)          | Forward: CGTGCTGCTTGGAGACGGG              |
|                                    | Reverse: TGCAGCGAATCTGTGAGGT               |
| ADAMTS3 (NM_172454.2)             | Forward: TGTTGAAGTGGTCGCTGG                |
|                                    | Reverse: AGCCAGGGGTCATATACAA              |
| ADAMTS4 (NM_010288.3)             | Forward: TGCCAGAGTACGCGCTGTG               |
|                                    | Reverse: AAGGTAGTGGTCCGTGTCG               |
| ADAMTS5 (NM_001159382.1)          | Forward: GAGCTAAGGGCCAGGCTAT             |
|                                    | Reverse: TGGCCGTACATCCAGTTCCT               |

**Quantitative real-time PCR**

The tissues and cells were prepared for total mRNA using TRIzol Reagent (15-596-018 Invitrogen/Life Technologies, USA) according to the protocol of manufacturer, and disrupted using mortar while dipping in liquid nitrogen. Complementary DNA was synthesized using One Step PrimeScript™ RT-PCR Kit (RR064A, Takara, Dalian, China). Quantitative real-time PCR was performed in quadruplicate on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, USA) using the TaqMan Gene Expression assays product (4448491, Applied Biosystems) and Fast SYBR Green Master Mix (4385610, Life Technologies). Gene of interest cycle thresholds were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) house-keeper level by the ΔΔCt method. The relative primer pairs were listed in Table 1.

**Immunofluorescence staining**

After the tissue sections were deparaffinized and hydrated as mentioned above, the antigen retrieval was carried out at 100°C for 20 min and cooled to RT. The sections were blocked in 5% bovine serum albumin (BSA) (05470, Sigma-Aldrich) for 1 h, and incubated with primary antibodies diluted in 1% BSA overnight at 4°C. Primary antibodies were purchased from Abcam (UK), including anti-F4/80 (ab100790, 1:100 dilution), anti-CD3 (ab5690, 1:100 dilution), anti-ADAMTS1 (ab39194, 1:200 dilution), anti-ADAMTS2 (ab125226, 1:200 dilution), and anti-ADAMTS5 (ab41037, 1:200 dilution). The information for antibodies is listed in Table 2. Specifically, anti-F4/80 is used as a marker for macrophages and anti-CD3 is for T cells, the fluorescence secondary antibody (Alexa Fluor 647, ab150079) was incubated (1:200) for 2 h, and sections were kept out
of light from this step on. Phosphate-buffered (PBS, P1010, Solarbio) was used to rinse the sections for 10 min, and 4,6-diamidino2-phenylindole (DAPI) was applied for 10 min to dye the nuclei. The images were captured with confocal laser scanning microscope (FV3000, Olympus Corporation, Japan).

**Statistical analysis**

The data was collected from at least three independent experiments, and then statistically analyzed via one-way analysis of variance. The difference was statistically significant when \( P < 0.05 \).

**Results**

**Degeneration and destruction of the articular cartilage in ACLT-induced OA mice**

At 4 weeks after ACLT surgery, significant degeneration occurred in the articular cartilage. The \( \mu \)CT showed obvious destruction on the surface of the articular cartilage of the knee joint, including the femoral head and tibial head. The volume of bone tissue relative to total tissue volume (BV/TV) of subchondral bone was lower than in the Sham group (Figure 1a). In contrast, the surface of articular cartilage in the Sham group was smooth and intact. However, not only was the surface of articular cartilage rough, but the meniscus was also worn and cracked in the ACLT group. In addition, the thickness of the articular cartilage layer and the chondrocyte number were obviously reduced, along with abnormal vacuolar changes and uneven cartilage matrix staining (Figure 1b). To further detect changes in cartilage matrix, we used safranin O-fast green and Masson’s trichrome stains. With safranin O, the proteoglycan was stained red, which showed a decreased amount of proteoglycan in the ACLT group, while the hypertrophic cartilage zone was enlarged (Figure 1c). Masson’s trichrome staining showed blue collagen fibers and decreased red-stained calcified cartilage in the ACLT group (Figure 1d). Masson’s trichrome stained collagen fibers, mucus, and cartilage blue, while the calcified cartilage was stained red, and the cell nuclei were dark blue.

**Enhanced inflammatory reaction and bone resorption in ACLT-induced OA**

An inflammatory reaction is one of the key features of osteoarthritis. The inflammatory reaction promotes degeneration and destruction of the articular cartilage and enhances bone resorption in the subchondral bone. By immunofluorescence, F4/80 (marker for macrophages) and CD3 (marker for T cells) were increased in the bone marrow of the femur at 4 weeks after surgery, indicating enhanced acute inflammation (Figure 2). The levels of IL-1\( \beta \) and TNF-\( \alpha \) in blood, as measured by ELISA, were also measured to further characterize inflammation. In the ACLT group, the concentration of IL-1\( \beta \) was approximately 1.80-fold in the Sham group, and TNF-\( \alpha \) was increased by approximately 1.35-fold (Figure 3a). Osteoclasts were red after TRAP staining. Osteoclasts were distributed on the surface of calcified bone tissue. Osteoclasts were increased in the ACLT group and were 2.4 times as numerous as in the Sham group (Figure 3b,c).

**mRNA expression of the ADAMTS family in bone-related tissues and cells**

By qPCR (Figure 4), the expression of ADAMTS5 was strongest in chondrocytes, osteoblasts, osteocytes, and cartilage. The expression of ADAMTS1 was strongest in cortical bone. Overall, the expression levels of ADAMTS1, ADAMTS2, and ADAMTS5 were the strongest among the ADAMTS1-5 family. Thus, it would be representative to analyze the ADAMTS family by detecting the expression of the ADAMTS1, ADAMTS2, and ADAMTS5 proteases in the knee joint of ACLT-induced OA mice.

**Table 2. Information of Antibodies in this study.**

| Antibody name | Description | Dilution | RRID number | Product number |
|---------------|-------------|----------|-------------|---------------|
| F4/80         | Rabbit polyclonal | 1/100    | AB_10675322 | ab100790      |
| CD3           | Rabbit polyclonal | 1/100    | AB_305055   | ab5690        |
| ADAMTS1       | Rabbit polyclonal | 1/200    | AB_2221876  | ab39194       |
| ADAMTS2/NPI   | Rabbit polyclonal | 1/200    | AB_10975350 | ab125226      |
| ADAMTS5       | Rabbit polyclonal | 1/200    | AB_2222327  | ab41037       |

*Abcam, Cambridge, UK.
ADAMTS1 expression in the knee joint of OA mice

The ADAMTS1 protease expression in the knee joint was assayed by immunofluorescence (Figure 5). We focused on three areas: the articular cartilage, the subchondral bone, and the growth plate. In articular cartilage, ADAMTS1 was weakly expressed in the Sham group and slightly increased in the ACLT group but was mainly located in the middle layer of articular cartilage (red-boxed area in Figure 5a, b). In the subchondral bone, ADAMTS1 was distributed in the extracellular space around bone cells, and its expression was significantly increased in the ACLT group (yellow-boxed area in Figure 5a,c). ADAMTS1 expression in the ACLT group also showed an increasing trend in the cartilage matrix of the growth plate (cyan-boxed area in Figure 5a,d).

Figure 1. OA phenotypes in the knee joint of mice at four weeks after ACLT. (a) Micro-CT the destruction of articular cartilage and the meniscus. The ratio of bone volume to tissue volume (BV/TV) was decreased in subchondral trabecular bone of the ACLT group. (b) HE staining showing OA joint changes. The articular cartilage layer was thinner, with fewer chondrocytes. Space between black arrows shows the thickness of articular cartilage. (c) Representative safranin O staining indicating proteoglycan loss in articular cartilage of the ACLT group (space between black arrows). (d) Representative Masson trichrome staining indicating the reduced content of collagen fibrils in the articular cartilage of the ACLT group (arrow). Scale bar = 200 µm.
**ADAMTS2 expression in the knee joint of OA mice**

The immunofluorescence images showed that ADAMTS2 was highly expressed in the ACLT group (Figure 6). ADAMTS2 was expressed in all layers of articular cartilage in the Sham group, while its expression significantly increased in the ACLT group (red-boxed area in Figure 6a,b). ADAMTS2 expression was also significantly increased in the subchondral bone (yellow-boxed area in Figure 6a,c) and the growth plate (cyan-boxed area in Figure 6a,d).

**ADAMTS5 expression in the knee joint of OA mice**

ADAMTS5 expression was increased not only in articular cartilage, subchondral bone, and growth plate but also in the meniscus (Figure 7). In articular cartilage, ADAMTS5 was expressed on the surface of articular cartilage in the Sham group but significantly increased in the ACLT group (red-boxed area in Figure 7a,b). In subchondral bone, ADAMTS5 was expressed at a low level in the Sham group, but its expression increased in the ACLT group (yellow-boxed area in Figure 7a,c). Similarly, ADAMTS5 significantly increased in chondrocytes and cartilage matrix of growth plates in the ACLT group (cyan-boxed area in Figure 7a,d).

**Discussion**

In this study, we successfully established an OA model via anterior cruciate ligament transection (ACLT) of the knee joints of C57BL/6 J mice. We showed the
symptoms of early-stage ACLT-induced OA from multiple aspects. It has been reported that ADAMTS1 and ADAMTS5 were significantly increased in the synovial fluid of OA patients [10], suggesting that the ADAMTS family may play a key role in osteoarthritis. However, there has been no detailed study on the expression and distribution of the ADAMTS family in ACLT-induced OA knee joints. Thus, our work will benefit future research since ACLT is a very common method for establishing an OA research model.

ADAMTS1 was first found in a gene screen of mouse tumors [11]. ADAMTS1 mRNA is expressed in both articular cartilage and growth plate cartilage of normal mice. It belongs to the aggrecanase family and has weak cartilage matrix degradation ability, and its substrates include other extracellular matrix proteins, such as versican, and type I collagen [12]. However, a previous study showed that aggrecan and degraded product contents of cartilage in ADAMTS1 knockout mice manifested no obvious difference compared with normal mice [13]. On the other hand, overexpression of ADAMTS1 could influence bone mineral density. In addition, ADAMTS1 is considered a potential mediator of bone remodeling [14]. ADAMTS1 is expressed in osteoblasts and upregulated where new bone is forming [15], which is consistent with our results. Another study showed that ADAMTS1 promotes type I collagen degradation and osteoblast proliferation [16]. According to our results, ADAMTS1 expression was increased in the subchondral bone of osteoarthritis mice, which may be related to abnormal bone resorption of subchondral bone in osteoarthritis patients.

As a procollagen N-proteinase, ADAMTS2 is involved in the transformation of procollagen into

![Figure 3. Osteoclasts are increased in OA. (a) The levels of IL-1β and TNF-α in serum were both increased, as detected by enzyme-linked immunosorbent assay (n = 5, *P < 0.05). (b and c) Tartrate-resistant acid phosphatase (TRAP) staining showing increased osteoclasts (red), indicating overactive bone resorption in subchondral bone (n = 3, *P < 0.05). Scale bar = 100 µm.](image-url)
collagen. Only processed procollagens can transform to normal collagen monomers and finally form normal collagen fibers [17]. ADAMTS2 is capable of cleaving procollagen types I, II and III. In addition, both TNF-α and TGF-β can promote the secretion of ADAMTS2. In our results, the expression of ADAMTS2 was significantly increased in articular cartilage, subchondral bone, and growth plates in osteoarthritis, indicating its significant role in osteoarthritis.

ADAMTS5, also named aggrecanase 2, the most efficient aggrecanases, mainly functions in degrading aggrecan, so it has attracted much attention in the exploration of the pathological mechanism in osteoarthritis. In a study on OA, it was found that ADAMTS5 was the

Figure 4. The mRNA levels of the ADAMTS family in bone-related tissues and cells. ADAMTS1, ADAMTS2 and ADAMTS5 had the highest expression. (a) mRNA expression of the ADAMTS family in chondrocytes and cartilage. (b) mRNA expression of the ADAMTS family in osteoblasts and cancellous bone. (c) mRNA expression of the ADAMTS family in osteocytes and cortical bone.
main aggrecan-degrading enzyme [18,19]. According to our results, ADAMTS5 was significantly enhanced in the articular cartilage of OA knee joints, which was consistent with its strong aggrecan degradation ability.

In previous studies, many efforts were made to determine the role of the MMP family in the pathological process of OA [20–23]. However, the degradation process in OA is highly complex and should not be analyzed only via the MMP family. Thus, we further explored the role of the ADAMTS family in OA, as it may take part in several physiological and pathological processes of OA due to its multiple functions. For this study, we determined the expression and distribution patterns of ADAMTS1, ADAMTS2, and ADAMTS5 in the knee joint of ACLT-induced OA mice. Our results will be helpful for in-depth studies of the roles and mechanisms of the ADAMTS

Figure 5. The expression and distribution of ADAMTS1 in the knee joint of OA mice. (a) Representative images showing the expression changes of ADAMTS1 in OA by immunofluorescence. The magnified boxed areas show the expression changes of ADAMTS1 in the articular cartilage (b), subchondral bone (c) and growth plate (d). White arrows show slight increase of ADAMTS1 protein expression. Dapi, 4’,6-diamidino-2-phenylindole; F, femur; M, meniscus; T, tibia. Scale bar =500 µm.
protease family in OA. Further research is still needed to determine the mechanisms of action of the ADAMTS family in the initial stage and the development of OA.

**Conclusion**

In this study, we established an OA research model by ACLT in mice and characterized its pathological phenotypes, including morphology and inflammation. In addition, we measured the mRNA levels of the ADAMTS family in bone-related tissues and cells both in vivo and in vitro. We further demonstrated that the protein levels of ADAMTS1, ADAMTS2, and ADAMTS5 were all upregulated in the articular cartilage, subchondral bone, and growth plate of OA mice.
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Disclosure statement
The authors reported no conflicts of interest related to this study.

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