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Identification of α_2-Macroglobulin as a Master Inhibitor of Cartilage-Degrading Factors That Attenuates the Progression of Posttraumatic Osteoarthritis

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Objective. **To determine if supplemental intra-articular α_2-macroglobulin (α_2M) has a chondroprotective effect in a rat model of osteoarthritis (OA).**

Methods. **Using Western blotting, mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry, α_2M was identified as a potential therapeutic agent through a comparison of α_2M concentrations in serum, synovial fluid (SF), and cartilage from normal subjects and patients with OA. In cultured chondrocytes, the effects of α_2M on interleukin-1 (IL-1)–induced cartilage catabolic enzymes were evaluated by Luminex assay and ELISA. In vivo effects on cartilage degeneration and matrix metalloproteinase 13 (MMP-13) concentration were evaluated in male rats (n = 120) randomized to 1 of 4 treatments: 1) anterior cruciate ligament transection (ACLT) and saline injections, 2) ACLT and 1 IU/kg injections of α_2M, 3) ACLT and 2 IU/kg injections of α_2M, or 4) sham operation and saline injections. Rats were administered intraarticular injections for 6 weeks. The concentration of MMP-13 in SF lavage fluid was measured using ELISA. OA-related gene expression was quantified by real-time quantitative polymerase chain reaction. The extent of OA progression was graded by histologic examination.**

Results. **In both normal subjects and OA patients, α_2M levels were lower in SF as compared to serum, and in OA patients, MMP-13 levels were higher in SF than in serum. In vitro, α_2M inhibited the induction of MMP-13 by IL-1 in a dose-dependent manner in human chondrocytes. In the rat model of ACLT OA, supplemental intraarticular injection of α_2M reduced the concentration of MMP-13 in SF, had a favorable effect on OA-related gene expression, and attenuated OA progression.**

Conclusion. **The plasma protease inhibitor α_2M is not present in sufficient concentrations to inactivate the high concentrations of catabolic factors found in OA SF. Our findings suggest that supplemental intra-articular α_2M provides chondral protection in posttraumatic OA.**

Anterior cruciate ligament (ACL) injury is one of the most frequent musculoskeletal injuries in adoles-

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cents and young adults, and it is known to place the injured knee at risk of early posttraumatic osteoarthritis (OA) (1). Evidence suggests that the current gold standard of treatment, surgical ACL reconstruction, does not appreciably reduce this risk (2–7). Discovery of mechanisms responsible for posttraumatic OA in this patient population would enable clinicians to identify markers and targets that could be used to aid in the diagnosis, treatment, and prevention of posttraumatic OA.

OA progression is due, at least in part, to the up-regulation of inflammatory mediators and proteases (8–11). Since elevated levels of catabolic enzymes in synovial fluid (SF) are associated with chondrocyte death and cartilage matrix degeneration within 1 week of injury (8,12–15), early intervention strategies should focus on modulating these cartilage-degrading enzymes within this time frame. Evidence from our group (11,16–19) and others (8,13,14) suggests that new molecular interventions targeting these enzymes can potentially arrest these adverse events and preserve joint health. It is unlikely; however, that blocking only one of these catabolic factors would be enough to repress posttraumatic OA after injury.

Our initial hypothesis was that endogenous serum protease inhibitors are not present in adequate amounts in the joint. The serum protease inhibitor α_2-macroglobulin (α_2M) was identified as a potential therapeutic agent through the screening of serum, SF, and cartilage from normal subjects and OA patients by Western blotting, mass spectrometry, enzyme-linked immunosorbent assay (ELISA), and immunohistochemistry. Alpha$_2$-macroglobulin inhibits all classes of endoproteases (20,21). Our hypothesis is that α_2M injected intraarticularly could potentially slow cartilage damage after traumatic knee injury by neutralizing cartilage catabolic enzymes. To establish the functional role of α_2M in OA development, concentrations of cartilage catabolic factors and their gene expression were quantified after α_2M supplementation in cultures of human OA chondrocytes as well as cartilage organ cultures. The changes in cartilage catabolic enzymes were monitored in vivo by fluorescence molecular tomography (FMT) using a mouse partial medial meniscectomy model of OA. To assess the effects of α_2M on cartilage damage in vivo, we used a rat model of ACL transection (ACLT) with supplemental intraarticular injections of α_2M shortly after injury. We also characterized the endogenous expression of α_2M in human knee joint tissues.

Our results strongly indicate that α_2M is a negative regulator of cartilage catabolic enzymes but that it is not present in vivo at sufficient levels to counteract the increased concentrations of catabolic factors that appear after injury. Therefore, supplemental intraarticular injection of α_2M shortly after injury may provide chondral protection to the ACL-injured knee by reducing catabolic enzymes.

MATERIALS AND METHODS

This study was approved by the Institutional Review Board and the Institutional Animal Care and Use Committee of the Rhode Island Hospital.

Human samples. OA cartilage samples were obtained from patients at the time of total joint arthroplasty (n = 17 [11 women and 6 men]; mean ± SD age 68.6 ± 8.6 years [range 55–79 years]). Normal cartilage samples were obtained from patients undergoing tumor resection (n = 6 [all men]; mean ± SD age 23.8 ± 13.6 years [range 15–51 years]). These samples were a subset of those obtained during a previous study (22). Serum and SF samples were also obtained prior to and during knee joint arthroplasty, respectively, in another set of OA patients (n = 39 [20 women and 19 men]; mean ± SD age 65.4 ± 9.6 years [range 48–80 years]). OA diagnosis was determined by clinician assessment according to the American College of Rheumatology (ACR) criteria (23). Normal serum samples (n = 43; mean ± SD age 37.5 ± 10.2 years [range 20–56 years]) were also collected.

Cartilage damage in knee joints was classified during arthroscopy before debridement or by direct surgical observation during joint replacement, using the Outerbridge cartilage damage score (scores 1 and 2 corresponded to early-stage disease and scores 3 and 4 corresponded to end-stage disease) (24). Normal SF samples were collected from the contralateral uninjured knees of patients undergoing unilateral ACL reconstruction, normal subjects undergoing arthroscopy, and one healthy volunteer (n = 33; mean ± SD age 26.3 ± 11.0 years [range 15–54 years]); these subjects had no history of knee injury and had normal findings on standing radiographs. Human cartilage samples were assessed and divided into categories: OA cartilage was severely fibrillated and from the more affected compartment (usually the medial compartment; Mankin score 9–14), and relatively normal or nonfibrillated cartilage was from the uninvolved compartment (usually the lateral compartment; Mankin score 0–2) (25).

Human serum and SF collection and analysis. Human serum and SF samples were divided into aliquots and frozen at −80°C until analysis. SF samples were treated with 15 units/ml of bovine testicular hyaluronidase before the experiments were performed, as described previously (22). Levels of α_2M and matrix metalloproteinase 13 (MMP-13) in human serum and SF were measured using ELISA.

Western blotting. Total proteins (14 μg) were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE; 10% polyacrylamide) under reducing conditions, as previously reported (22). The membrane was probed with an antibody against α_2M (1:1,000 dilution). Horseradish peroxidase–conjugated secondary antibody IgG (heavy and light chains) (Bio-Rad) was diluted 1:3,000. Enhanced chemiluminescence (Amersham) was used to visualize immu-

noreactive proteins. Alpha$_1$-antitrypsin was used as a loading control.

Chondrocyte isolation and primary culture. Human chondrocytes were isolated as previously described (22) and plated either in 8-well chambers at 1×10^5 cells/well or in 6-well culture plates at 1×10^6 cells/plate. At 90% confluence, the cells were cultured overnight under serum-free conditions and then treated with 10 ng/ml recombinant human interleukin-1β (IL-1β) for 2 hours before treatment with different concentrations of the α_2M protein (Sigma). Culture medium was collected after 24 hours and analyzed for the presence of catabolic cytokines and MMPs. The same experiments were also performed using the human chondrocyte cell line C-28/I2 and cartilage tissues (26).

Luminex assay. A Luminex Human Inflammatory 5-Plex Panel (Invitrogen) was used to measure levels of granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-1β, IL-6, IL-8, and tumor necrosis factor α (TNFα). A Luminex Human MMP 3-Plex Panel (Invitrogen) was used to measure levels of MMPs 3, 9, and 13. The 5-plex or 3-plex beads were incubated with 100 μl of either standard or samples for 2 hours. Biotinylated antibodies were added and incubated for 1 hour. After washing, R-phycoerythrin–labeled streptavidin was added and the plates were analyzed using a Luminex xMAP instrument. The concentration of MMP-13 activity in the medium was quantified using ELISA (R&D Systems). APMA activates any potentially active forms of MMP-13 present in a sample. Since we intended to measure the endogenous levels of active, but not inactive, MMP-13 in samples, we did not add APMA to the sample wells.

Mouse partial medial meniscectomy model of OA. The mouse partial medial meniscectomy model of OA was used to determine the kinetics of inflammatory mediators (27), since FMT imaging in our facility can only be used for mice. Cathepsin is a family of proteases. The changes in cathepsin-mediated inflammation in vivo were monitored by FMT at different time points after meniscectomy (n = 4).

Fluorescence molecular tomography. FMT is a noninvasive and quantitative fluorescence-based technology with high molecular specificity and sensitivity for 3-dimensional tissue imaging of live animals. Biologic processes can be probed dynamically at different time points (from hours to days) (28,29). ProSense 750 fluorescent agents are activated by cathepsins B, L, and S and plasmin, but are undetectable in the inactivated state. Mice were injected with ProSense 750EX and imaged using FMT (VisEn) 24 hours after injection.

The rat ACLT model of OA and treatment with supplemental intraarticular α_2M. One hundred twenty 10-week-old rats (180–230 gm each) were randomized to 1 of 4 groups (n = 30 per group): 1) ACLT and saline injections, 2) ACLT and 1 IU/kg injections of α_2M, 3) ACLT and 2 IU/kg injections of α_2M, and 4) sham operation and saline injections. ACLTs and sham operations were performed on the right knees, as described previously (18). Alpha$_2$-macroglobulin was dissolved in 20 μl of saline to treat rats in the 1 IU/kg and 2 IU/kg groups. Intraarticular injections were performed immediately following and 3 days after ACLT, and then weekly for 6 weeks. Animals in the 2 saline injection groups were administered an equivalent volume of saline in their right knees at identical time points to control for any procedural effects. All animals were euthanized 8 weeks after the operation. In each group, 15 rats were studied histologically and 15 were assessed using real-time polymerase chain reaction (PCR).

Rat SF collection and analyses. SF lavage was collected as described previously (11). MMP-13 content was measured in the SF samples using ELISA according to the instructions of the manufacturer (Uscn Life Science). Colorimetric density on the developed plates was determined using a microplate reader (Model BF10000; Packard) set to 450 nm. ELISA analysis of each sample was performed in duplicate.

Real-time quantitative PCR (qPCR). The cartilage samples were ground with a mortar and pestle under liquid nitrogen, and total RNA was isolated from human and rat knee joint cartilage using an RNeasy isolation kit (Qiagen) (22). Cartilage samples (tibial plateau and femur condyle) from 3 rats were dissected using a scalpel and pooled together; there were 5 pooled samples per group. Total RNA (1μg) was reverse transcribed to complementary DNA (cDNA) using an iScript cDNA synthesis kit (Bio-Rad). The resulting cDNA (40 ng/μl) was used as the template to quantify the relative content of messenger RNA (mRNA) using a QuantiTect SYBR Green PCR kit (Qiagen) with a DNA Engine Opticon 2 Continuous Fluorescence Detection System (MJ Research). Primer pairs were as follows: for human α_2M, CTT-TCC-TTG-ATG-ACC-CAA-GCG-CC (forward) and GTT-GAA- AAT-AGT-CAG-CGA-CCT (reverse); for rat Col2a1, AAG-GGA-CAC-CGA-GGT-TTC-ACT-GG (forward) and GGG-CCT-GTT-TCT-CCT-GAG-CGT (reverse); for rat Acan, CAG-TGC-GAT-GCA-GGC-TGG-CT (forward) and CCT-CCG-GCA-CTC-GTT-GGC-TG (reverse); for rat Col10a1, CCA-GGT-GTC-CCA-GGA-TTC-CC (forward) and CAA-GCG-GCA-TCC-CAG-AAA-GC (reverse); for rat Mmp3, TTG-TCC-TTC-GAT-GCA-GTC-AG (forward) and AGA-CGG-CCA-AAA-TGA-AGA-GA (reverse); for rat Mmp13, GGA-CCT-TCT-GGT-CTT-CTG-GC (forward) and GGA-TGC-TTA-GGG-TTG-GGG-TC (reverse); for rat Runx2, CCG-CAC-GAC-AAC-CGC-ACC-AT (forward) and CGC-TCC-GGC-CCA-CAA-ATC-TC (reverse); and for 18S RNA, CGG-CTA-CCA-CAT-CCA-AGG-AA (forward) and GCT-GGA-ATT-ACC-GCG-GCT (reverse). Relative transcript levels were calculated according to the equation $x = 2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \Delta C_t\,E - \Delta C_t\,C$ ($\Delta C_t\,E = C_t$exp $- C_t$18S, $\Delta C_t\,C = C_t C - C_t$18S) (22).

Histologic assessment. Gross morphologic lesions on the femoral condyle and tibial plateau in rats (n = 15 per group) were visualized by India ink staining (30). The femurs and tibiae were hemisected in the midsagittal plane, and each half was embedded in a single block of Paraplast X-tra (Fisher). Blocks were trimmed to expose cartilage. Ten adjacent sections were collected at intervals of 0 μm, 100 μm, and 200 μm. Two serial 6-μm–thick sections from each interval were stained with Safranin O. Cartilage degradation was quantified using the Osteoarthritis Research Society International (OARSI) grading system (31). Three independent observers scored each section under blinded conditions, and the scores for all of the sections cut from the medial and lateral tibial plateaus were averaged within each joint.

Immunohistochemistry. Specimens were stained with India ink and analyzed by immunohistochemistry using a Histostain-SP kit (Invitrogen) in order to detect type II collagen, type X collagen, and MMP-13. The sections were digested with 5 mg/ml of hyaluronidase in phosphate buffered

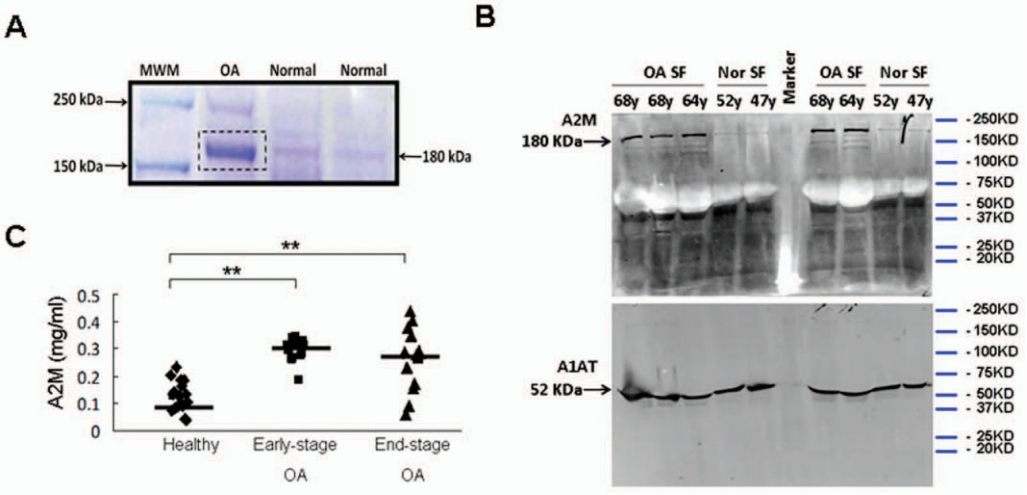

Figure 1. Levels of α_2-macroglobulin (α_2M [A2M]) are elevated in osteoarthritis (OA) synovial fluid (SF) as compared to normal (Nor) SF. **A,** Proteins in SF were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide). Staining with Coomassie blue showed a more prominent band (~180 kd) in OA SF (from a 64-year-old man) as compared to normal SF (from a 52-year-old man and a 50-year-old man). Sequencing of this band using mass spectrometry showed that 37 of the unique peptides matched α_2M. MWM = molecular weight marker. **B,** Expression of α_2M was further assessed by Western blotting, which demonstrated higher α_2M expression in OA SF (from 2 68-year-old men and a 64-year-old woman) as compared to normal SF (from a 52-year-old man and a 47-year-old man). Alpha$_1$-antitrypsin (α_1AT) was used as a loading control. **C,** Alpha$_2$-macroglobulin content in SF from healthy individuals (n =16), patients with early-stage OA (n = 18), and patients with end-stage OA (n =14) was quantified by enzyme-linked immunosorbent assay. Each data point represents a single subject; horizontal lines show the mean. ** = $P < 0.01$. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38576/abstract.

saline (PBS; Sigma) for 20 minutes. Nonspecific protein binding was blocked by incubation with a serum blocking solution (Li-Cor). The sections were incubated with 2 μg/ml of antibody against either rat type X collagen (EMD Biosciences), MMP-13, or type II collagen at 4°C overnight. Thereafter, the sections were treated sequentially with biotinylated secondary antibody and streptavidin–peroxidase conjugate and then developed in 3,3′-diaminobenzidine chromogen.

To detect the distribution of α_2M in human cartilage and synovial membrane, 6-μm sections were analyzed by immunofluorescent staining with a polyclonal antibody against α_2M. The sections were incubated with primary antibody at 4°C overnight. After washing, affinity-purified tetramethylrhodamine isothiocyanate–conjugated donkey anti-goat secondary antibody (1:500 dilution; Jackson ImmunoResearch) was applied with 0.5 mg/ml of Hoechst nuclear dye (Pierce). The negative control sections were incubated with isotype control antibody (sc-8514-P) in PBS.

Statistical analysis. Analyses of variance were used to compare the in vitro concentrations of α_2M, cartilage catabolic factors (GM-CSF, IL-1β, IL-1 receptor antagonist, IL-6, IL-8, TNFα, MMP-3, MMP-9, and MMP-13) in different groups, and the in vivo concentrations of MMP-13 and levels of mRNA for Col2a1, Acan, Mmp3, Mmp13, Runx2, and Col10a1. A 2-way mixed absolute intraclass correlation coefficient for the cartilage damage score was calculated. Followup pairwise comparisons between multiple experimental groups were carried out with orthogonal contrasts using Scheffe's test (α = 0.05) and a test of homogeneity. Adjusted P values for the multiple comparisons were reported. P values less than 0.05 were considered significant. Statistical analyses were performed using SPSS software.

RESULTS

Identification of α_2M in the SF of OA patients and normal controls. SDS-PAGE gel stained with Coomassie blue showed a more prominent band (~180 kd) in OA SF than in normal SF (Figure 1A). The band was further analyzed by mass spectrometry. The top 4 candidate proteins, α_2M, fibronectin, apolipoprotein B, and complement component C3, were chosen for further analysis. The increase in α_2M levels was validated in OA SF (n = 3) as compared to normal SF (n = 2) using Western blotting. Alpha$_1$-antitrypsin was used as loading control (16) (Figure 1B).

ELISA results showed that the concentration of α_2M was higher in SF from patients with early-stage OA (n =18; mean ± SD 0.302 ± 0.04 mg/ml) (Outerbridge score 1–2) and patients with end-stage OA (n =14; 0.264 ± 0.11 mg/ml) (Outerbridge score 3–4) as compared to healthy subjects (n =16; 0.126 ± 0.06 mg/ml) (Outerbridge score 0) (Figure 1C). There was an obvious difference between α_2M concentrations in OA SF versus normal SF when assessed using Western blotting, while ELISA resulted in a 3-fold difference.

Alpha$_2$-macroglobulin expression in human cartilage and synovial membrane. Positive staining of α_2M in cartilage and synovium from OA patients and normal controls was seen on immunohistochemical analysis

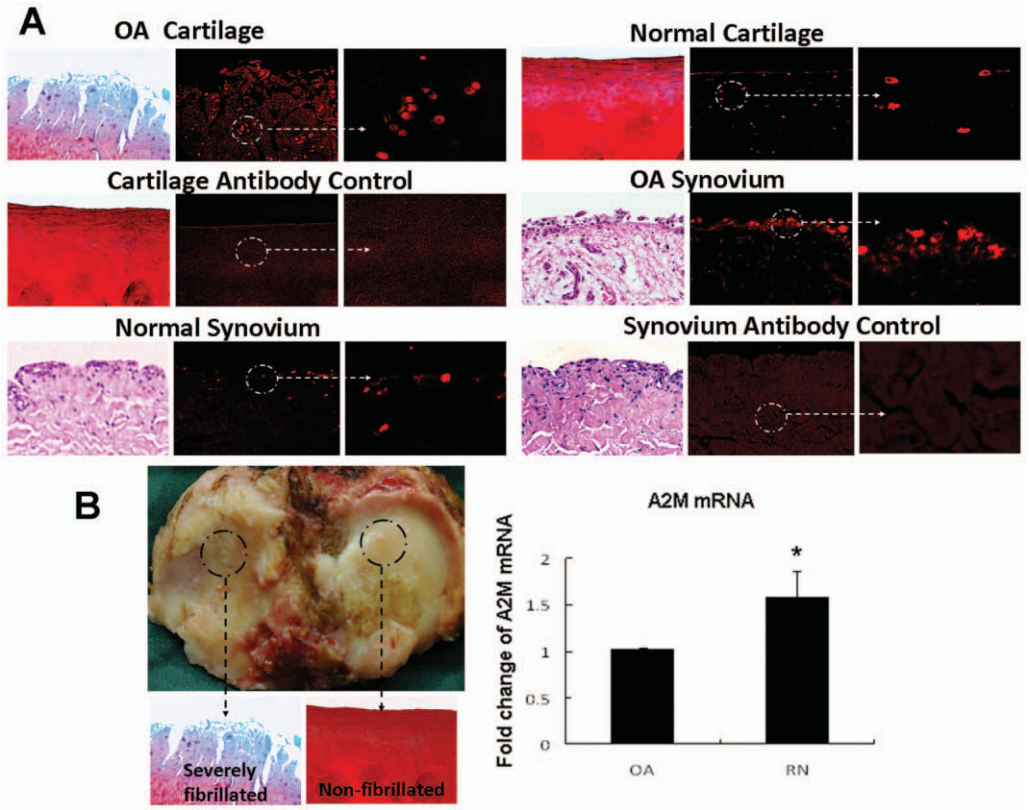

Figure 2. Alpha$_2$-macroglobulin is expressed in cartilage and synovium. **A,** Positive staining of α_2M (red fluorescence) was observed in OA cartilage and OA synovium (n = 5 patients; mean ± SD age 64.8 ± 8.7 years [range 55–77 years]), as well as in normal cartilage and normal synovium (n = 6 subjects; mean ± SD age 23.8 ± 13.6 years [range 15–51 years]), indicating that α_2M was produced in joint tissue. Cartilage sections were stained with Safranin O, and synovium samples were stained with hematoxylin and eosin. The right panels are higher-magnification views of the circled areas in the middle panels. **B,** Total RNA was isolated from severely fibrillated OA cartilage (Mankin score 9–14) and the adjacent relatively normal (RN) cartilage (i.e., nonfibrillated cartilage; Mankin score 0–2) from the same OA patients (n = 7; mean ± SD age 73.7 ± 7.3 years [range 58–79 years]). Representative Safranin O–stained cartilage sections from the circled areas in the top left panel are shown in the bottom left panel. Real-time polymerase chain reaction results shown in the right panel demonstrate that α_2M mRNA levels were lower in OA cartilage as compared to relatively normal cartilage from the same patient. Values are the mean ± SEM. * = $P < 0.05$ versus OA cartilage. See Figure 1 for other definitions. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38576/abstract.

(Figure 2A). Quantification of mRNA from knee joints of OA patients indicated that α_2M mRNA levels were lower in cartilage from the involved compartment as compared to the uninvolved compartment (relatively normal cartilage) of the joint from the same patient (n = 7 patients) (Figure 2B). Our data showed that α_2M is synthesized de novo by chondrocytes and synovial membrane. However, OA chondrocytes appeared to have reduced ability to produce α_2M as compared to chondrocytes in the adjacent relatively normal cartilage (Figure 2B).

Lower α_2M levels in SF than in normal control serum and OA serum, and elevated MMP-13 levels in OA SF. We compared the protein levels of α_2M in SF and serum and found that, although α_2M levels were higher in OA SF as compared to normal SF, the levels were much lower in OA SF than in OA serum (Figure 3A). We further found that α_2M protein expression was the reverse of MMP-13 protein expression in the serum and SF of OA patients (α_2M expression in serum mean ± SEM 1.53 ± 0.052 mg/ml and α_2M expression in SF 0.24 ± 0.002 mg/ml [$P = 0.002$] as compared to MMP-13 expression in serum 91.07 ± 16.12 ng/ml and MMP-13 expression in SF 251.01 ± 19.23 ng/ml [$P = 0.007$]; n = 20) (Figure 3A).

Suppression of catabolic cytokines and MMPs by α_2M. ELISA results demonstrated that exogenous α_2M inhibited the induction of MMP-13 activity by IL-1 in a dose-dependent manner in human primary OA chondrocytes, in human OA cartilage explants, and in the

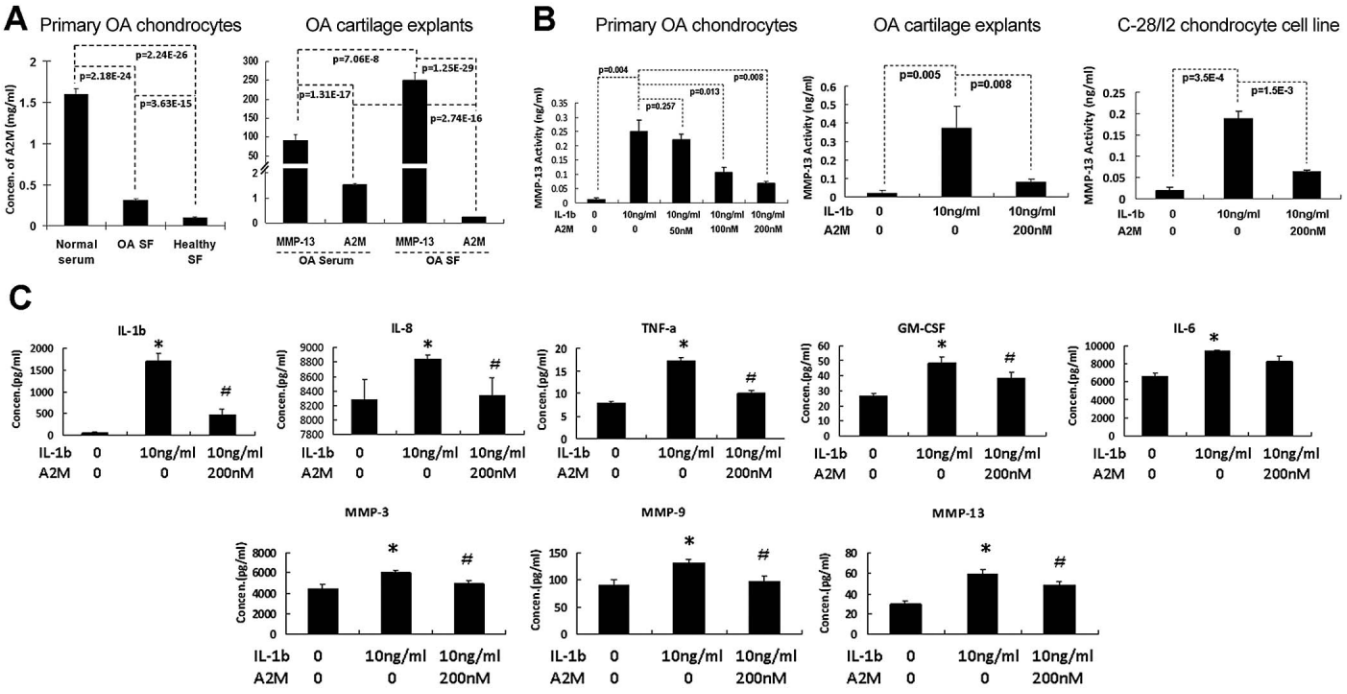

Figure 3. Cartilage catabolic cytokines and matrix metalloproteinases (MMPs) are negatively regulated by α_2M. **A,** Left, Although α_2M concentrations (concen.) were higher in OA SF (n = 39; mean ± SD age 65.4 ± 9.6 years [range 48–80 years]) than in normal SF (n = 33; mean ± SD age 26.3 ± 11.0 years [range 15–47 years]), the levels of α_2M in SF were much lower than in normal serum (n = 43; mean ± SD age 37.5 ± 10.2 years [range 20–56 years]). Right, Higher concentrations of α_2M and lower levels of MMP-13 were detected in serum from OA patients as compared to OA SF from the same patients (n = 20; mean ± SD age 67.0 ± 7.1 years [range 55–79 years]). **B,** MMP-13 activity in human OA chondrocytes was induced by interleukin-1 (IL-1) (10 ng/ml), and inhibited by α_2M in a dose-dependent manner. The most potent inhibition was achieved with 200 n*M* α_2M. Similarly, IL-1–induced MMP-13 activity was inhibited by α_2M (200 n*M*) in human OA cartilage explant cultures and in the C-28/I2 human chondrocyte cell line. **C,** IL-1β (10 ng/ml) induced the expression of IL-8, tumor necrosis factor α (TNFα), granulocyte–macrophage colony-stimulating factor (GM-CSF), IL-6, and MMPs as well as IL-1β itself in human OA chondrocytes, while α_2M (200 n*M*) inhibited the IL-1β–induced increases in all but the IL-6 levels. Human OA chondrocytes and explants were collected from the same patients (n = 5; mean ± SD age 65.2 ± 8.1 years [range 58–79 years]). Values are the mean ± SEM. $* = P < 0.05$ versus controls; $\# = P < 0.05$ versus IL-1 alone. See Figure 1 for other definitions.

human chondrocyte cell line C-28/I2 (26) (Figure 3B). Our data from the Luminex Human Inflammatory Panel and Luminex Human MMP Panel further demonstrated that treatment of human primary OA chondrocytes with α_2M decreased protein levels of the majority of cartilage catabolic cytokines and enzymes induced by IL-1β, including IL-1β, IL-8, TNFα, GM-CSF, MMP-3, MMP-9, and MMP-13 (Figure 3C). Thus, these results suggest that α_2M supplementation beyond endogenous levels may inhibit OA cartilage degradation in vivo by decreasing cartilage catabolic and inflammatory factors, in addition to inhibiting protease activity.

Peak in joint cathepsin/plasmin activity on day 2 in the mouse partial medial meniscectomy model of OA. To determine the optimal timing for administering exogenous α_2M, characteristics of cathepsin were determined in a mouse model. FMT data indicated that the strongest cathepsin-mediated joint inflammation occured 2 days after surgery (Figure 4A). This model resulted in histologically and radiographically evident OA 9 weeks after surgery (Figure 4B).

Attenuation of posttraumatic OA pathogenesis using supplemental intraarticular α_2M in a rat model of ACLT. We found a significant decrease in the OA score in α_2M-treated rats as compared with rats that underwent ACLT and saline treatment. After treatment with α_2M at either concentration, stronger Safranin O staining, more cellularity but less chondrocyte cloning, and less fibrillation were observed than in the saline-treated groups. Cartilage from rats administered the 2 IU/kg injection of α_2M had stronger staining and more intact surface than did cartilage from rats administered the 1

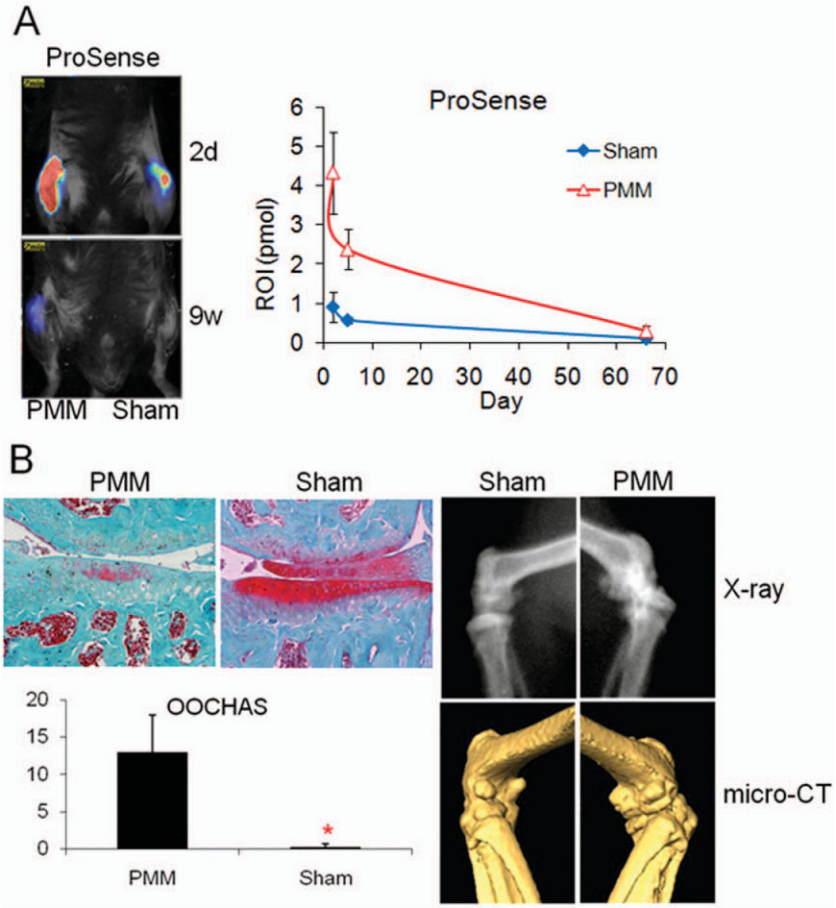

Figure 4. Cathepsin levels peak 2 days after knee joint injury. **A,** The highest levels of cathepsin, as detected by fluorescence molecular tomography of mice 9 weeks after partial medial meniscectomy (PMM), were observed 2 days after surgery, indicating an early catabolic response that subsided thereafter. The mean ± SD region of interest (ROI) signal intensities (n = 4) at each time point over a 9-week period are shown at the right. **B,** Safranin O staining and quantification of the histologic results using the Osteoarthritis Research Society International Cartilage Histopathology Assessment System (OOCHAS) indicated articular cartilage damage and loss of proteoglycan staining. Radiography and micro–computed tomography (micro-CT) demonstrated the morphologic changes in the entire knee joint at 9 weeks. Bars show the mean ± SD. * = $P < 0.05$ versus partial medial meniscectomy group. Color figure can be viewed in the online issue, which is available at http://onlinelibrary.wiley.com/doi/10.1002/art.38576/abstract.

IU/kg injection of α_2M, but weaker staining than that from control rats that underwent sham operation (Figure 5A). OARSI histologic grading system scores in both α_2M-treated groups suggested mild degeneration (mean ± SD 14.1 ± 4.2 in the 1 IU/kg group and 8.8 ± 3.9 in the 2 IU/kg group; $P = 0.001$), while cartilage damage in rats that underwent ACLT and saline treatment was significantly more severe (19.9 ± 1.8; $P < 0.01$) (Figure 5B). The cartilage from rats that underwent sham operation had the least amount of damage (0.2 ± 0.2; $P < 0.01$).

Histologic changes were evaluated at 8 weeks only. Type II collagen staining in both groups of α_2M-treated rats was stronger than that in rats that received ACLT and saline injection (Figure 5C), and dose dependency was evident. In addition, there was less immunostaining for MMP-13 and type X collagen in the animals treated with 2 IU/kg of α_2M as compared to those treated with 1 IU/kg of α_2M (Figure 5C). Cartilage damage was associated with a change in the levels of MMP-13 in joint lavage fluid. In rats that underwent ACLT and saline treatment, the mean ± SD MMP-13 level in joint lavage fluid was 2,450.67 ± 789.21 ng/ml, which was higher than that in rats administered the 1 IU/kg injection of α_2M (604.35 ± 198.76 ng/ml; $P = 0.035$), rats administered the 2 IU/kg injection of α_2M

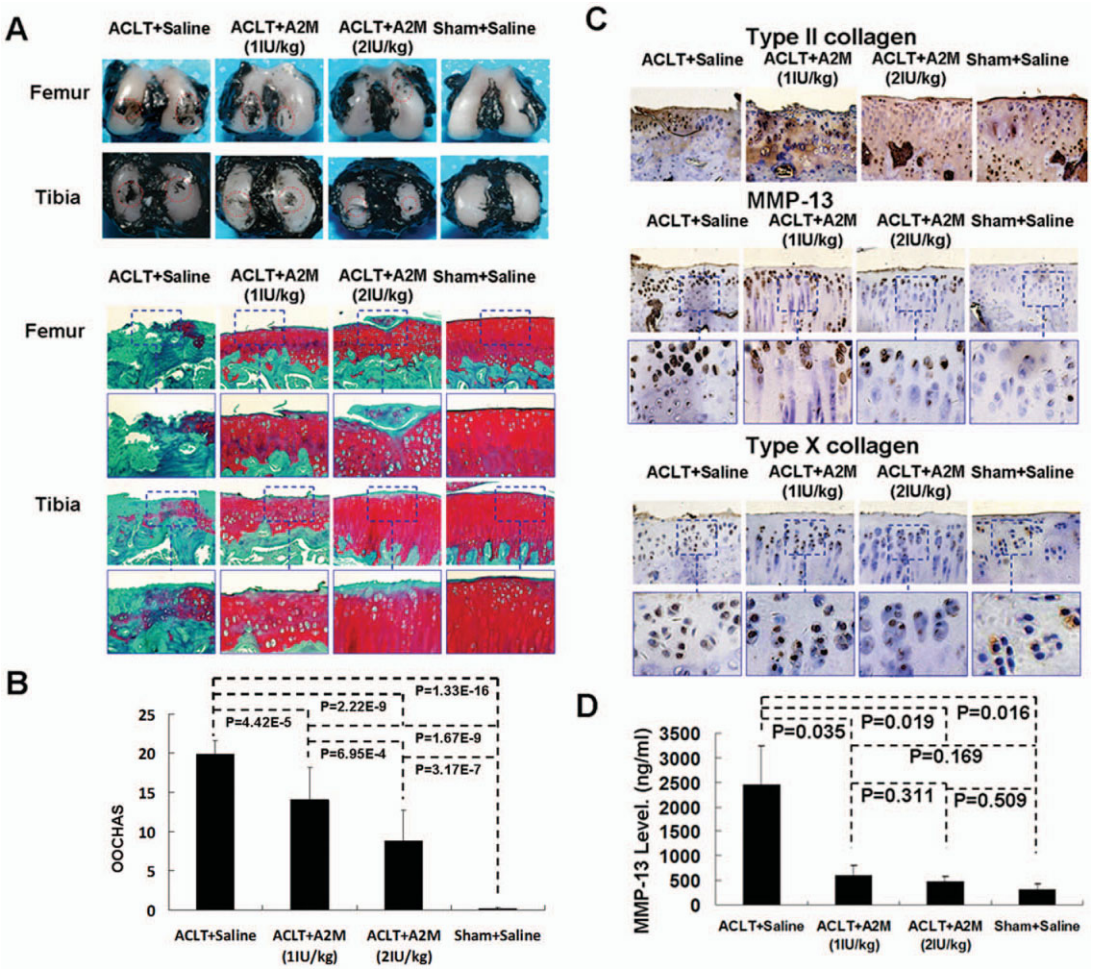

Figure 5. Supplemental intraarticular α_2M attenuates the pathogenesis of posttraumatic OA in a rat model of anterior cruciate ligament transection (ACLT). **A,** Decreased India ink staining and a smoother surface with stronger Safranin O staining were detected in the articular cartilage of α_2M-treated animals as compared to untreated controls. **B,** The Osteoarthritis Research Society International Cartilage Histopathology Assessment System (OOCHAS) score indicated that cartilage damage was most severe in rats that underwent ACLT and saline treatment, while cartilage in rats that underwent sham operation had the least damage. Cartilage damage was also reduced in rats that received the 2 IU/kg dose of α_2M as compared to rats that received the 1 IU/kg dose of α_2M. Values are the mean ± SD. **C,** Type II collagen expression in articular cartilage was higher in the α_2M-treated and the sham-operated rats than in rats that underwent ACLT and saline treatment. In contrast, matrix metalloproteinase 13 (MMP-13) and type X collagen staining was elevated in rats that underwent ACLT and saline treatment, but was lower in the α_2M-treated and sham-operated rats, which is consistent with reduced OA damage in these rats. **D,** In α_2M-treated rats, the concentration of MMP-13 in SF was lower than that in rats that underwent ACLT and saline treatment and was similar to that in sham-operated rats. Values are the mean ± SD. In **A** and **C,** the bottom panels are higher-magnification views of the boxed areas in the top panels. See Figure 1 for other definitions.

(464.23 ± 110.07 ng/ml; $P = 0.019$), and rats that underwent sham operation (312.52 ± 129.13; $P = 0.016$) (Figure 5D).

Enhancement of matrix gene expression in cartilage by α_2M. Real-time qPCR results indicated that supplemental intraarticular α_2M enhanced the levels of mRNA for Col2a1 and Acan, and suppressed the levels of mRNA for Mmp3, Mmp13, Runx2, and Col10a1 in the rat model of ACLT (Figure 6). Col2a1 mRNA levels in rats that underwent ACLT and saline treatment were significantly lower than those in rats that underwent ACLT and were administered 1 IU/kg or 2 IU/kg of α_2M and those in rats that underwent sham operation, and there was no significant difference among the latter 3 groups. Acan mRNA levels in rats treated with 2 IU/kg of α_2M and rats that underwent sham operation and saline treatment were significantly higher than the levels in rats that underwent ACLT and saline treatment. In contrast, levels of

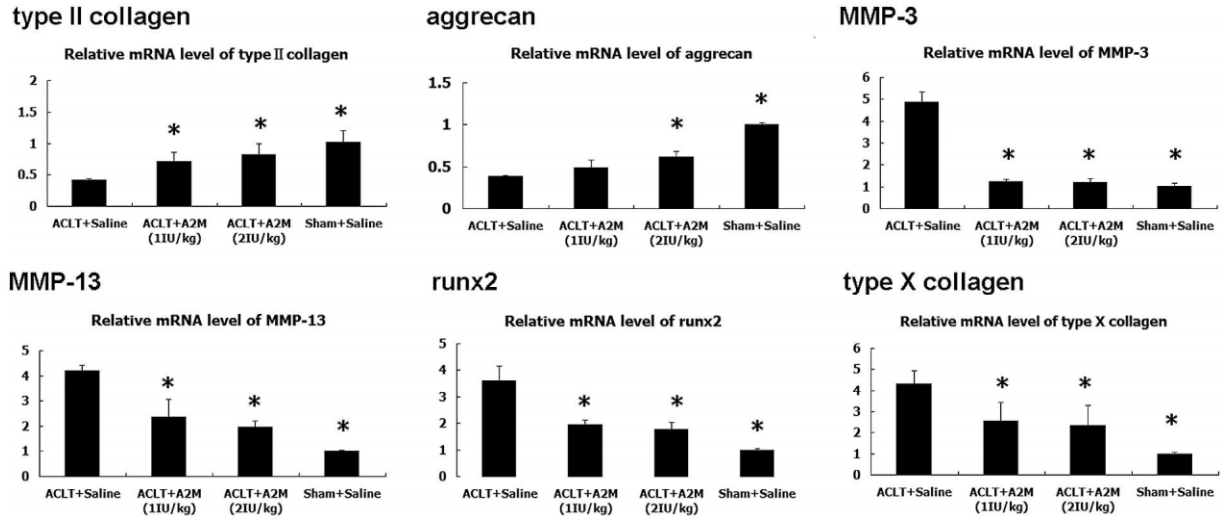

Figure 6. Supplemental intraarticular α_2-macroglobulin (α_2M [A2M]) inhibits catabolism and enhances anabolic metabolism in a rat model of anterior cruciate ligament transection (ACLT). Levels of mRNA for type II collagen and aggrecan were increased in rats that were administered 1 IU/kg or 2 IU/kg of α_2M as compared to rats that underwent ACLT and saline treatment, suggesting that α_2M has a positive impact on anabolic metabolism. In contrast, matrix metalloproteinase 3 (MMP-3), MMP-13, Runx2, and type X collagen showed the opposite pattern. These genes were expressed at a lower level in rats that were administered 1 IU/kg or 2 IU/kg of α_2M as compared to rats that underwent ACLT and saline treatment. Values are the mean ± SEM. $* = P < 0.01$ versus the ACLT and saline treatment group.

mRNA for Mmp3, Mmp13, Runx2, and Col10a1 in rats that underwent ACLT and saline treatment were the highest among the 4 groups. These data suggest that α_2M has a chondroprotective effect in vivo by decreasing gene expression of catabolic factors and hypertrophic markers, as well as by increasing anabolic gene expression.

DISCUSSION

The results of this study suggest that α_2M is a powerful inhibitor of many cartilage catabolic factors and that it can attenuate posttraumatic OA cartilage degeneration. Alpha$_2$-macroglobulin, a major protease inhibitor, is produced by the liver, with serum concentrations of 2.2–2.3 mg/ml. We have shown that α_2M is also produced by chondrocytes and synoviocytes, although the levels in SF are lower than those in serum (Figures 1 and 2). We found that higher levels of α_2M are present in the serum as compared to the SF of normal subjects and OA patients. This difference is thought to be due to the large molecular weight of α_2M, which prevents it from migrating into the SF (32,33).

Since α_2M inhibits all classes of endoproteases (20,21), it could be used to slow the development of posttraumatic OA by neutralizing cartilage catabolic factors. Studies have shown that α_2M inhibits ADAMTS-4, ADAMTS-5, ADAMTS-7, ADAMTS-12 (20,21), and MMP-13 activity (34). Thus, the protease/α_2M balance may play an important role in mediating cartilage destruction that occurs because of catabolic factors. We found that concentrations of MMP-13 were 2.8-fold higher in human OA SF samples than in serum, but α_2M levels were 7-fold lower in human OA SF samples than in serum (Figure 3A). MMP-3 and IL-1β concentrations are also higher in OA SF as compared to serum by a factor of ~10 (35–37).

We have also shown that exogenous α_2M decreases these cartilage catabolic cytokines and enzymes in vitro (Figures 3B and C). In vivo data from FMT imaging confirmed that peak levels of the cartilage catabolic enzymes cathepsins B, L, and S and plasmin could be detected on day 2 after joint injury in the mouse model (Figure 4). Since elevated levels of catabolic enzymes in SF appear to induce chondrocyte death and cartilage matrix degeneration within 1 week of injury (8,12–15), early intervention may be critical for preventing or minimizing the development of posttraumatic OA. Our in vivo results in a rat model of ACLT suggest that this is true. Early supplemental intraarticular injection of α_2M reduced the level of MMP-13 in SF and attenuated the loss of cartilage proteoglycans and collagen erosion (Figures 5 and 6). Therefore, α_2M, a negative regulator of catabolic cytokines and enzymes, is likely a therapeutic candidate (20,21). The level of α_2M in normal SF is 0.126 mg/ml. One inhibitor unit is equal

to 0.048 mg of α_2M, increasing the α_2M concentration by 38% (assuming the rat joint contains 1 ml of SF). Future studies will focus on optimizing the dosing strategy.

Previous studies have demonstrated that α_2M binds cytokines, such as IL-1β and TNFα, and also enters into cells to regulate cellular responses to other growth factors and cytokines (38–40). Although the exact mechanism by which supplemental intraarticular α_2M attenuates cartilage degeneration is not clear, it is very likely that α_2M acts by binding cytokines in addition to directly neutralizing enzyme activities (20,41,42). The relative contributions of these mechanisms will be addressed in future studies.

A limited number of studies have attempted to indirectly quantify active α_2M by measuring conversion of total α_2M to inactive α_2M. In one study, 90% of α_2M was active in plasma; however, neutrophils and free radicals are capable of inactivating α_2M. Total α_2M in SF was less than α_2M in serum (41). During joint inflammation or sepsis, α_2M becomes inactive, presumably by complexing to proteinases (41,43). This suggests that from a therapeutic perspective, adequate supplemental α_2M would be needed to quench catabolic enzymes. We did not directly analyze SF for inactive versus active α_2M, since currently available reagents only recognize total α_2M.

A potential limitation of this study is that surgical transection of the ACL may not be as traumatic as an ACL injury sustained during physical activity. Bone bruises and chondral lesions frequently occur in the latter, and these concomitant injuries may also play a role in the development of posttraumatic OA. Nonetheless, the animal model of ACLT has been frequently used to study OA, and it mimics human OA both macroscopically and biochemically (11,44). Minimizing local joint inflammation until ACL reconstruction is performed may be an important preventive measure that could forestall the long-term development of posttraumatic OA. Another limitation of our study is the use of specimens from patients who were not age matched. Obtaining age-matched controls is challenging for studies of human OA. Therefore, α_2M analyses were performed using relatively normal cartilage and OA cartilage from the same patient. We recognize that the regions in which cartilage appears normal in the OA joint may not be entirely normal and that this cartilage is also exposed to high levels of cartilage catabolic factors in the OA SF (45). However, this method provides us with a reasonable benchmark for comparison, since it is tissue with minimal damage, and biologic variability is minimalized.

In summary, up-regulation of cartilage catabolic cytokines and enzymes is thought to be a key mechanism of cartilage damage. Thus, inhibition of these molecules will likely slow or prevent the progression of disease. Our novel data indicate that α_2M is a master inhibitor of many types of cartilage-degrading enzymes and that it acts not only by blocking activity, but also by decreasing gene expression and protein levels in the joint. The innate levels of α_2M in SF may not be sufficient to reduce the activities of catabolic enzymes that are present after joint injury. In this study, supplemental intraarticular injection of α_2M attenuated cartilage degeneration in a rat model of ACLT, suggesting that it may be a potential novel therapy for posttraumatic OA.

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AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. L. Wei had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Wang, X. Wei, Zhou, Chen, Terek, Fleming, Goldring, Ehrlich, G. Zhang, L. Wei.

Acquisition of data. Wang, X. Wei, Zhou, Chen, Terek, Fleming, Goldring, Ehrlich, G. Zhang, L. Wei.

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