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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 intraarticular 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 intraarticular 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

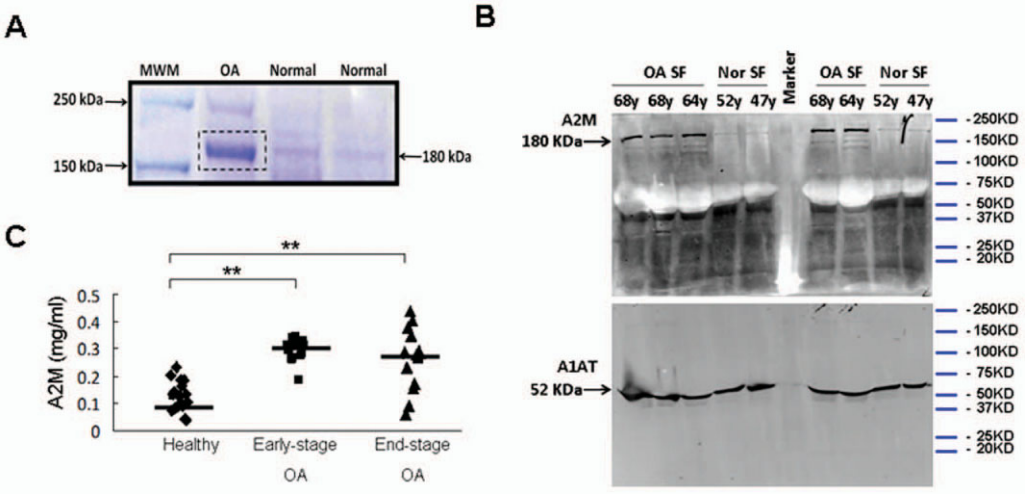

Figure 1. Levels of α_2-macroglobulin (α_2M [A2M]) are elevated in osteoarthritis (OA) synovial fluid (SF) as compared to normal (Nor) SF. **A,** Proteins in SF were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (10% polyacrylamide). Staining with Coomassie blue showed a more 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

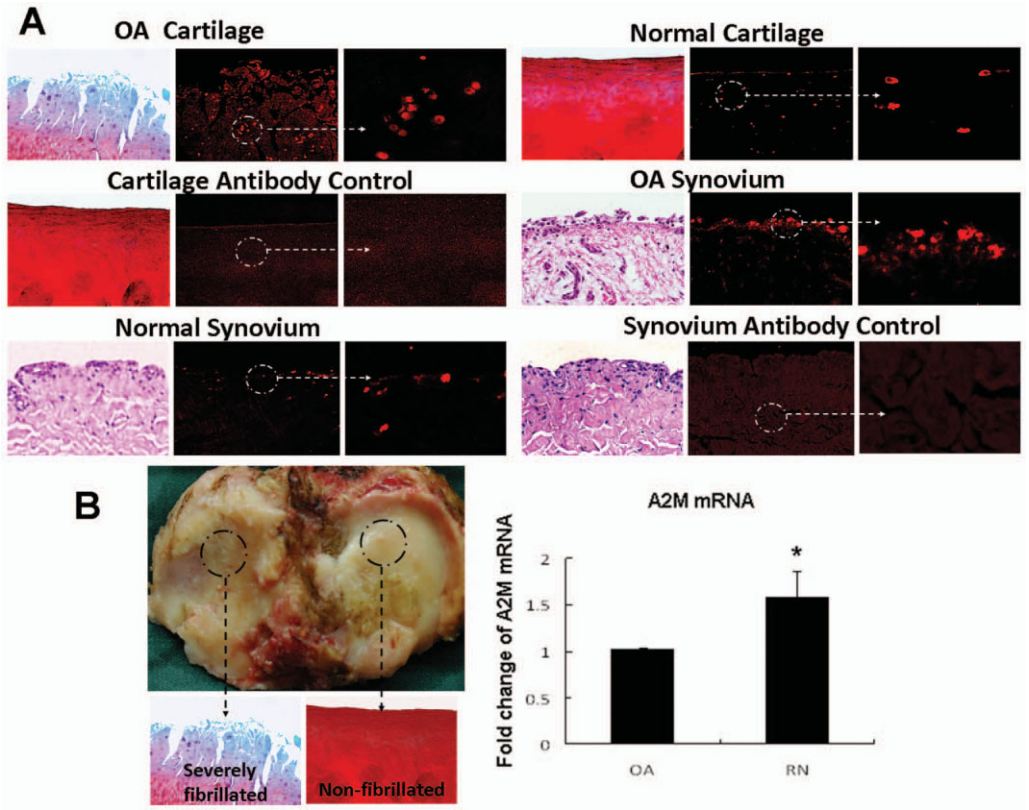

Figure 2. Alpha$_2$-macroglobulin is expressed in cartilage and synovium. **A,** Positive staining of α_2M (red fluorescence) was observed in OA cartilage and OA synovium (n = 5 patients; mean ± SD age 64.8 ± 8.7 years [range 55–77 years]), as well as in normal cartilage and normal synovium (n = 6 subjects; 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

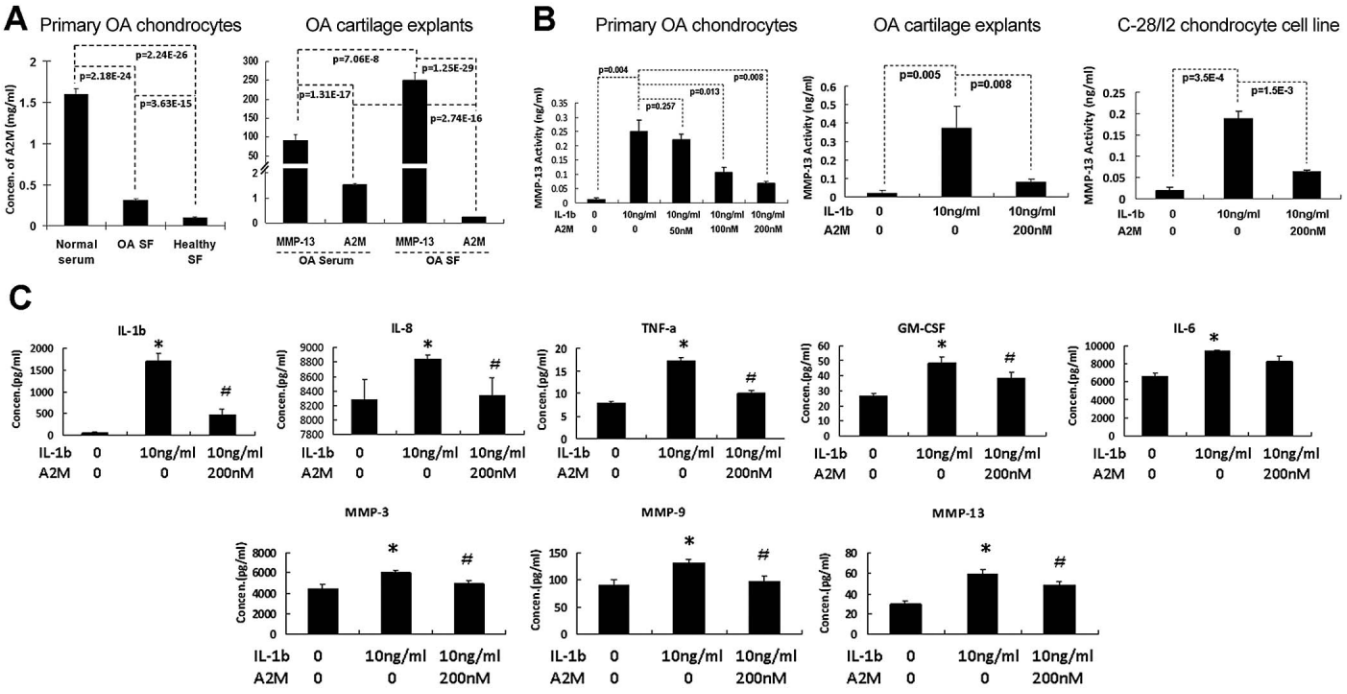

Figure 3. Cartilage catabolic cytokines and matrix metalloproteinases (MMPs) are negatively regulated by α_2M. **A,** Left, Although α_2M concentrations (concen.) were higher in OA SF (n = 39; mean ± SD age 65.4 ± 9.6 years [range 48–80 years]) than in normal SF (n = 33; mean ± SD age 26.3 ± 11.0 years [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

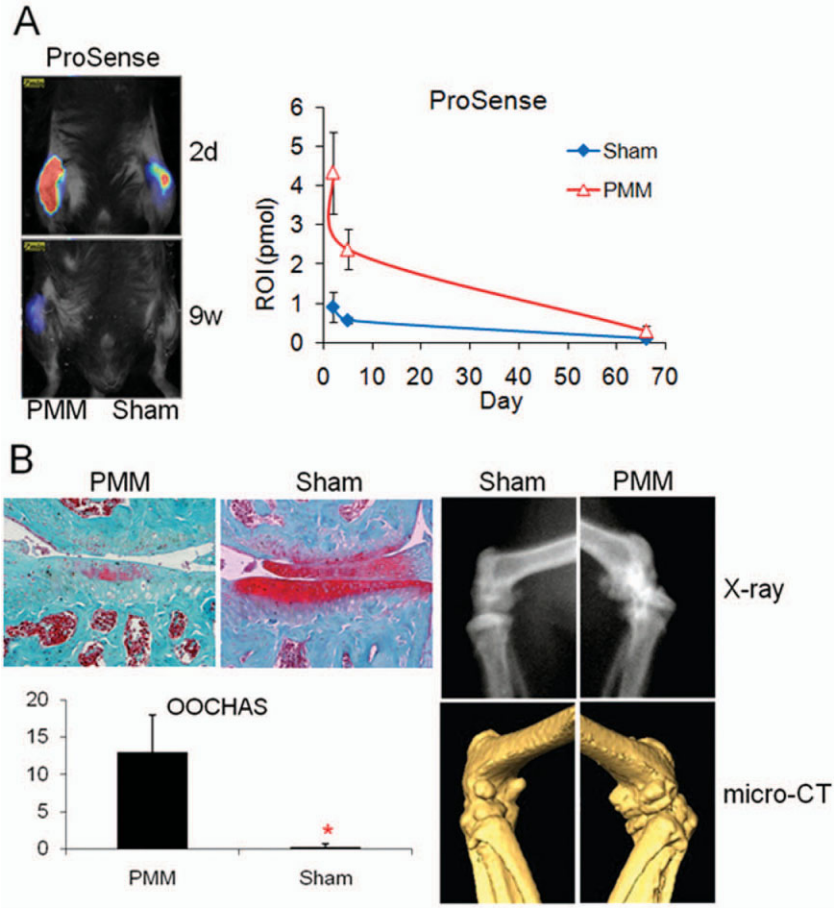

Figure 4. Cathepsin levels peak 2 days after knee joint injury. **A,** The highest levels of cathepsin, as detected by fluorescence molecular tomography of mice 9 weeks after partial medial meniscectomy (PMM), were observed 2 days after surgery, indicating an early catabolic response that subsided thereafter. 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

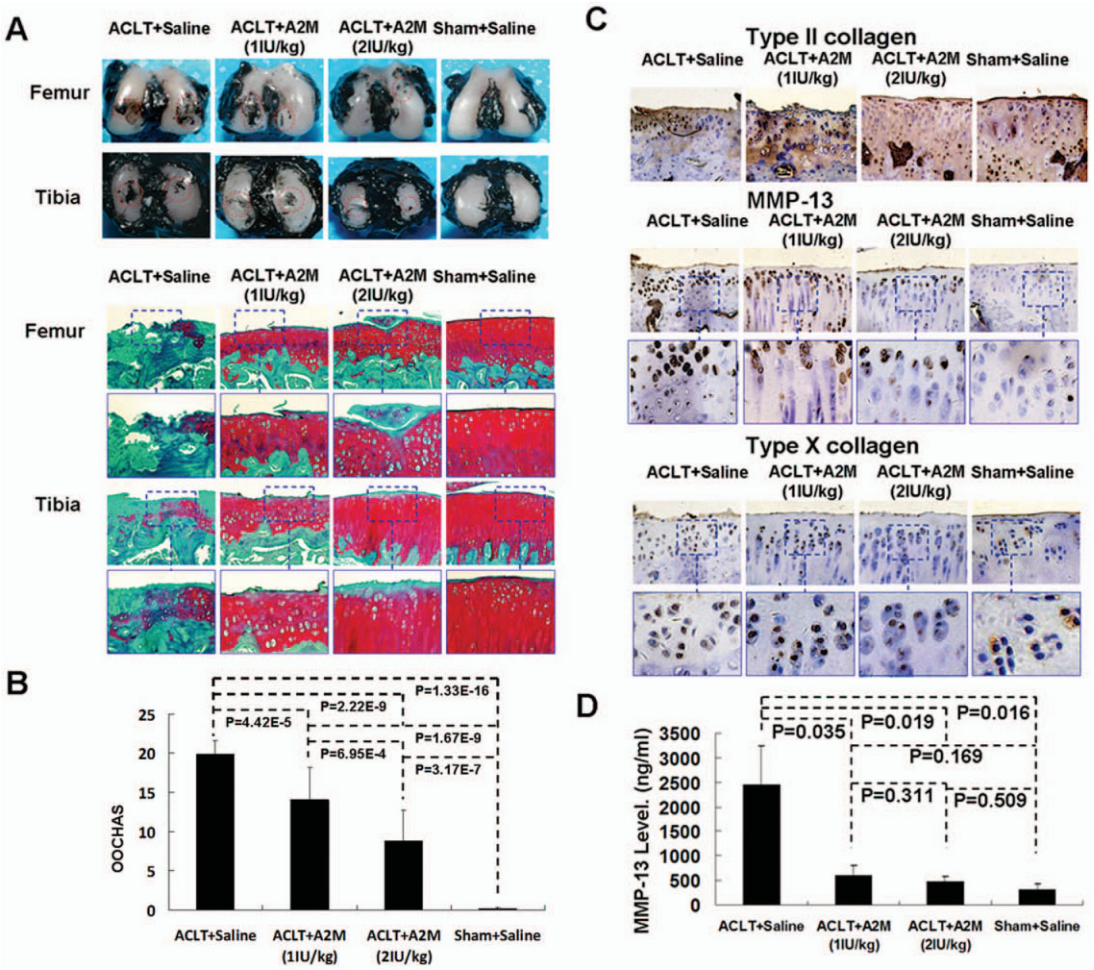

Figure 5. Supplemental intraarticular α_2M attenuates the pathogenesis of posttraumatic OA in a rat model of anterior cruciate ligament transection (ACLT). **A,** Decreased India ink staining and a smoother surface with stronger Safranin O staining were detected in the articular cartilage of α_2M-treated 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

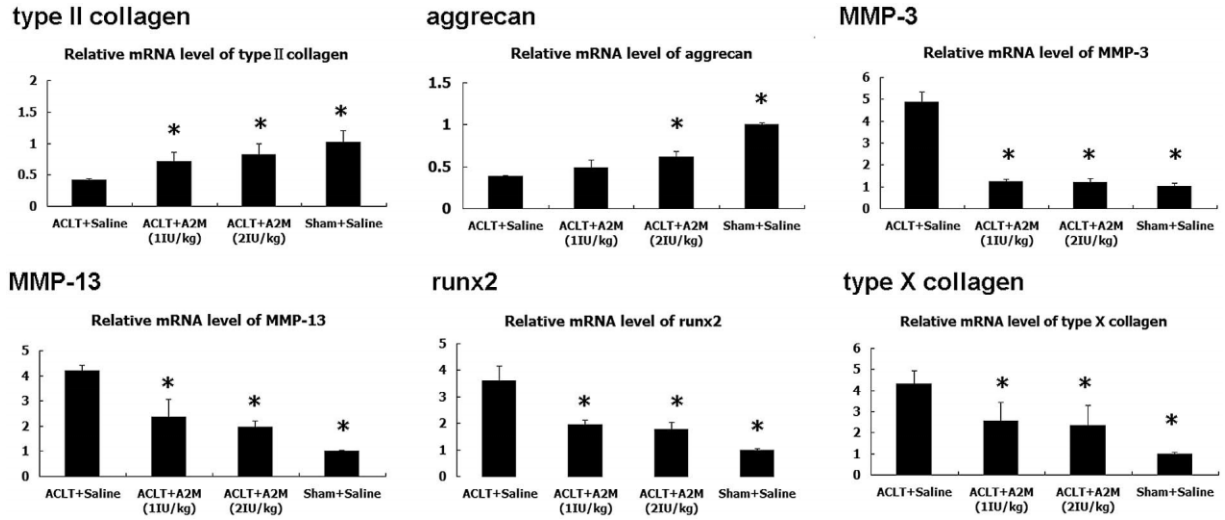

Figure 6. Supplemental intraarticular α_2-macroglobulin (α_2M [A2M]) inhibits catabolism and enhances anabolic metabolism in a rat model of anterior cruciate ligament transection (ACLT). Levels of mRNA for type II collagen and aggrecan were increased in rats that were administered 1 IU/kg or 2 IU/kg 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.

Analysis and interpretation of data. Wang, X. Wei, Zhou, J. Zhang, Li, Chen, Terek, Fleming, Goldring, Ehrlich, G. Zhang, L. Wei.

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State of the Art

Amniotic Fluid: Not Just Fetal Urine Anymore

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Amniotic fluid (AF) is a complex substance essential to fetal well-being. This article reviews recent discoveries and the current understanding of the origin and circulation of AF and its nutritive, protective, and diagnostic functions. Future directions for AF research are also discussed.
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INTRODUCTION

Amniotic fluid (AF) is a marvelously complex and dynamic milieu that changes as pregnancy progresses. AF contains nutrients and growth factors that facilitate fetal growth, provides mechanical cushioning and antimicrobial effectors that protect the fetus, and allows assessment of fetal maturity and disease. This article will review the development, content, and clinical significance of AF and its essential role in helping the fetus become a newborn.

DEVELOPMENT OF AF AND THE AF CIRCULATION

A fluid-filled extracelomic cavity which will eventually become the amnionic space is identified near the time of implantation, even before the embryo is recognizable. During embryogenesis, AF volume increases faster than embryonic size. The water in AF originally comes from maternal plasma and passes through the fetal membranes based on hydrostatic and osmotic forces. As the placenta and fetal vessels develop, water and solute from maternal plasma pass across the placenta to the fetus and then to the AF. In the early fetal period, AF volume and fetal size are related in a linear fashion. AF volume increases from about 25 ml at 10 weeks to about 400 ml at 20 weeks. During this period, AF composition is similar to fetal plasma. There is rapid bi-directional diffusion between the fetus and the AF across the not-yet-keratinized fetal skin and the surfaces of the amnion, placenta, and umbilical cord, each being freely permeable to water and solutes. During this phase of pregnancy, the AF serves both as a physiologic buffer and an extension of the fetal extracellular compartment. By 8 weeks of gestation, the urethra is patent and the fetal kidneys make urine. Shortly thereafter fetal swallowing begins; however, neither fetal urination nor swallowing contributes significantly to the content or volume of AF until the second half of pregnancy. Keratinization of fetal skin begins at 19 to 20 weeks of gestation and is usually complete at 25 weeks after conception. When keratinization is complete, the relationship between fetal size and AF volume is no longer linear. By 28 weeks of gestation, AF volume reaches a volume of $\sim$800 ml where it plateaus near term gestation and thereafter declines to $\sim$400 ml at 42 weeks.[1]

After the fetal skin is fully keratinized, AF volume is determined by factors that comprise the AF circulation. Five pathways of exchange have been identified between the amniotic space and the surrounding tissues (see Figure 1). Production of AF is predominately accomplished by excretion of fetal urine ($\sim$300 ml/kg fetal weight/day or 600 to 1200 ml/day near term) and the secretion of oral, nasal, tracheal, and pulmonary fluids ($\sim$60 to 100 ml/kg fetal weight/day).[2] Fetal breathing movements contribute to the efflux of lung fluid into the AF, but about half of the effluent is swallowed rather than entering the AF. While volume changes with each fetal breath are small, $<$5 ml per breath, and fetal breathing occurs only for 20 to 30 min of each hour in late gestation, the overall contribution of fetal breathing to AF volume is significant. Removal of AF is predominately accomplished by fetal swallowing ($\sim$200 to 250 ml/kg fetal weight/day). Additionally, a significant intramembranous pathway transfers fluid and solutes from the amniotic cavity to the fetal circulation across the amniotic membranes.[3] The human amnion is a single layer of epithelial cells separating the amniotic cavity from the vascularized chorion. Early in gestation these amniocytes are flattened, but as pregnancy progresses they become cuboidal and have increasing numbers of microvilli on their apical surface. Tortuous intercellular channels exist between the tight junctions of amniocytes. The amount of fluid that passes through the intramembranous pathway is highly variable and has been estimated at 200 to 500 ml/day.[4] The transmembranous pathway, the movement of AF across the fetal membranes and into the maternal circulation within the lining of the uterus, affects AF volume only minimally. While this process has not been directly measured, it is estimated to be $\sim$10 ml/day at term.[2] Sherer[5] is an excellent review of AF dynamics.

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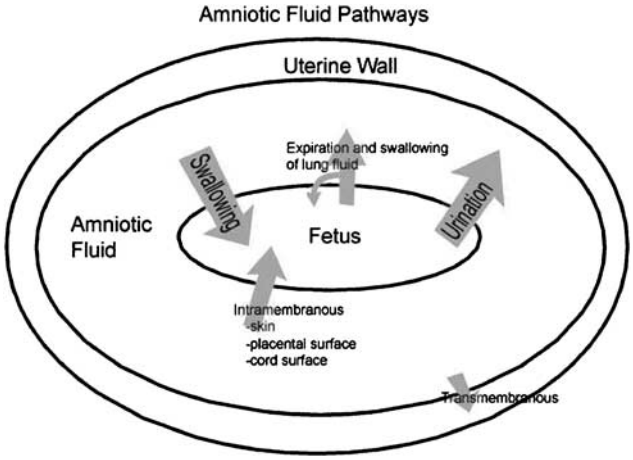

Figure 1. Amniotic fluid pathways.

The relative stability of AF volume in spite of large fluid shifts suggests that control mechanisms exist. It is noteworthy that only half of fetuses with esophageal atresia, and two-thirds of fetuses with duodenal or proximal jejunal atresia develop polyhydramnios; this suggests that other mechanisms besides swallowing are involved in AF volume regulation. Compensation via the intramembranous pathway is the best explanation for the significant number of fetuses with upper intestinal atresia who do not develop polyhydramnios. Compensation is evident in sheep where ligation of the esophagus leads to increased absorption of AF into the fetal circulation with no change in the total AF volume. Intramembranous absorption occurs against a hydrostatic gradient and had been assumed to be driven by passive diffusion due to an osmotic gradient. More recent studies show that passive diffusion accounts for only part of the intramembranous fluid absorption and that many solutes diffuse in the opposite direction (from fetus to AF). It is likely that much larger shifts of fluid and solutes occur by bulk transfer of AF with all of its dissolved solutes into the fetal circulation perhaps via a trans-cellular vesicular transport mechanism.[6] Vascular endothelial growth factor (VEGF) in the ovine fetal membranes appears to be a mediator of this process. VEGF promotes blood vessel development within the amnion and influences the permeability of the microvessels, which perfuse the fetal and placental surfaces.[7] The demonstration of aquaporin proteins in fetal membranes suggests the possibility of water channels as another potential regulator.[8]

Hormonal changes may also play a role in AF volume regulation. There are not significant numbers of receptors for estrogen or progesterone in fetal membranes after early pregnancy. Receptors for decidual prolactin, however, are widely expressed by both fetal and maternal tissues with increasing numbers as pregnancy progresses. There is evidence that decidual prolactin has an effect on amniotic permeability, although this is probably not the only hormonal or growth factor-related mechanism.[9]

Uterine perfusion also impacts AF volume. Maternal dehydration leads to increased fetal plasma osmolality and increased fetal production of arginine vasopressin. This causes an increase in the osmolality of both fetal urine and to a lesser extent AF. When arginine vasopressin is injected directly into ovine AF, fetal urine and AF osmolality increase and fetal urine output decreases significantly, and yet AF volume does not change suggesting reverse intramembranous flow from the isotonic fetal circulation to the hypertonic AF.[10]

The composition of AF changes with gestational age. In the second half of pregnancy, there is a decrease in sodium and chloride concentrations, an increase in urea and creatinine concentrations, and an overall decrease in AF osmolality. Many studies suggest that AF composition is more highly regulated than AF volume. Wintour and Shandley[4] has an excellent summary of these studies.

NUTRITIVE FUNCTIONS OF AF

AF contains carbohydrates, proteins and peptides, lipids, lactate, pyruvate, electrolytes, enzymes, and hormones. Prior to keratin production in fetal skin, amino acids diffuse from the placenta through the placental membranes into AF and from the fetal circulation through the fetal skin into AF. Later in pregnancy diffusion through the placental membranes persists and is augmented by fetal urinary excretion of amino acids.[11] Like breast milk, AF is rich in taurine which is found in greater quantity in AF than in maternal serum, while most other amino acids have lower concentrations in AF than in maternal and fetal blood. Glutamine is an essential precursor for nucleic acid biosynthesis in all cells and is particularly important in rapidly dividing cells such as intestinal mucosa cells. In fetal sheep, the uptake of glutamine from the AF by the fetal intestine is an active process.[12] Arginine also plays an essential role in fetal and placental development. Arginine is hydrolyzed to ornithine, which is then converted into the polyamines, putrescine, spermine, and spermidine, which are key regulators of placental angiogenesis, trophoblast growth, and embryogenesis. In sheep, the concentrations of arginine, ornithine, and polyamines increase rapidly in both allantoic and amniotic fluids early in gestation and remain elevated in AF throughout pregnancy. As gestational age increases, the swallowed polyamines in AF support proliferation and differentiation of intestinal epithelial cells.[13]

The role of swallowed carbohydrates and lipids in AF is less well defined. Growth-restricted rabbit fetuses were given infusions of dextrose or dextrose with amino acids directly into AF, and there was no improvement in growth, while an infusion of bovine AF did improve organ and somatic growth.[14] In a fetal rabbit model with esophageal ligation, the infusion of graded amounts of glucose or glucose with amino acids into AF enhanced organ weights and fetal

growth.[15] No studies have yet demonstrated reversal of fetal growth restriction (FGR) by intra-amniotic infusion of nutrient solutions.

Ligation of the esophagus in fetal rabbits to prevent swallowing followed by infusion of various solutions into the gut distal to the ligature has been performed to demonstrate the nutritive value of fetal swallowing. Those animals infused with lactated Ringer's solution had poor gut development whereas those infused with bovine AF showed more normal gut maturation.[16] Improved fetal organ growth with esophageal infusions of AF has also been shown in fetal sheep.[17] Trophic effects of AF have further been demonstrated on cultured human fetal small intestinal cells.[18] These studies suggest that growth factors found in AF, comparable to those in human milk, play a role in fetal growth and development. These trophic mediators are discussed below.

High levels of *epidermal growth factor* (EGF) are found in human milk and AF but not in standard infant formula. The concentration of EGF in amniotic fluid is four-fold higher than that found in fetal urine suggesting that the site of production is the amnionic membranes. EGF in human AF increases significantly during the second trimester, but is reduced in FGR. The function of EGF in the human fetus is largely unknown. In monkeys, in utero treatment with EGF improves lung maturity.[19] In fetal rabbits, enteral infusions of EGF reverse the effects of esophageal ligation.[16] EGF injected into the AF of pregnant rabbits increases small intestinal length and lactase and maltase activity compared to controls.[20] EGF receptors are present in the human stomach from the 18th week of gestation onward.

Transforming growth factor alpha (TGF-α) has a structure similar to EGF and binds to the same receptor. TGF-α is present in AF and human milk and, like EGF, is found in higher concentrations in human milk from women delivering prior to 27 weeks of gestation compared to those delivering after 27 weeks.[21] TGF-α is also produced in the fetal intestine. Recombinant TGF-α elicits a synergistic trophic response on cultured intestinal cells when combined with recombinant EGF, insulin-like growth factor-1, fibroblastic growth factor, and hepatocyte growth factor, but the trophic response is not as strong as either AF or breast milk.[18] The amnion cells of the umbilical cord express EGF, TGF-α, and the functional EGF/TGF-α receptor suggesting the possibility of a regulating role of the amnion in fetal growth and development. EGF and TGF-α have also been shown to stimulate the production of surfactant components.

Transforming growth factor beta-1 (TGF-β1) is found in rat AF and human breast milk, but is found in human AF only during the late stages of gestation. TGF-β1 is believed to induce terminal differentiation of intestinal epithelial cells and to accelerate the rate of healing of intestinal wounds by stimulating cell migration. TGF-β1 may also stimulate IgA production. Thus, TGF-β1 may prepare the fetal intestine for the extrauterine environment that is experienced after parturition at term.

Insulin-like growth factor I (IGF-I) is found in human milk and AF. When infused into the esophagus of fetal sheep, IGF-I improves somatic growth, spleen weight, and bowel wall thickness compared to control.[22] A single injection of labeled IGF-I into ovine AF demonstrated sustained delivery of IGF-I from the AF to the fetal gut and then into the systemic circulation over a 7-day period.[23] IGF-I and IGF-II receptors, as well as insulin receptors, are found throughout the human neonatal gut. IGF-I in AF may also increase the uptake of swallowed glutamine by the ovine gut.[12]

Erythropoietin (EPO) is found in human AF, colostrum, and mature milk. In the neonatal rat, enteral EPO is absorbed, stimulates erythropoiesis, and is a trophic factor for intestinal growth. The role of swallowed EPO in the human fetus and neonate is not clear. It is puzzling that concentrations of EPO are significant in AF and actually increase in human milk with the length of breast feeding, yet EPO is not absorbed from the gastrointestinal tract even though it is protected from digestion in the stomach.[24] This suggests the possibility of a local intestinal effect.

Granulocyte colony-stimulating factor (G-CSF) is found in human AF. When given enterally to suckling mice G-CSF enhances intestinal growth, suggesting that swallowed G-CSF in AF, colostrums, and breast milk may act as a topical growth factor in the fetal and neonatal intestine.

PROTECTIVE ROLE OF AF

AF plays an important protective role by providing a supportive cushion allowing fetal movement and growth. The oligohydramnios sequence and its related fetal deformations demonstrate the importance of this protective cushion.

AF also has a significant defensive role as a part of the innate immune system. The innate immune system is the first line of defense against pathogens and includes anatomic and physiologic barriers, enzymes and antimicrobial peptides, as well as phagocytosis and release of proinflammatory mediators by neutrophils and macrophages. Many of the substances that comprise the innate immune system have been identified in AF and vernix and have been shown to have significant antimicrobial properties; these include the α-defensins [HNP1-3], lactoferrin, lysozyme, bactericidal/permeability-increasing protein, calprotectin, secretory leukocyte protease inhibitor, psoriasin [S100A7], and a cathelicidin [LL-37].[25–27] These potent antimicrobials show broad-spectrum activity against bacteria, fungi, protozoa, and viruses. Perhaps the most important of these are the α-defensins [HNP1-3], which are found in significant concentrations in AF of women without evidence of infection and likely originate from the fetal skin and lung. AF concentrations of HNP1-3 increase with preterm labor, preterm premature rupture of membranes (PPROM), and chorioamnionitis probably due to release from neutrophils.

Lactoferrin (LF) is a glycoprotein with two binding sites for ferric ion. LF is found in human milk and appears in human AF at 20 weeks gestation increasing in concentration with gestation. Elevated levels of LF have been noted with preterm labor and with amnionitis. In pregnancies complicated by intra-amniotic infection (IAF), LF is likely secreted by neutrophils in the AF and by amniotic cells. LF has both bacteriostatic activity, due to sequestration of iron which is then unavailable for microbial growth, and bacteriocidal activity, due to binding to bacterial outer membranes triggering release of lipopolysaccharide. Enzymatic digestion of LF at acid pH releases a potent cationic, microbicidal peptide called lactoferricin. Lactoferricin shows antimicrobial effects against viruses, protozoa, and fungi.[28] Lactoferrin levels decrease with the onset of term labor.

The activity of the ''cellular'' innate immune system within AF as a protective mechanism for the fetus is less well defined. The numbers of mononuclear phagocytes (i.e., monocytes, macrophages, histiocytes) in AF are limited in normal pregnancies, while their numbers are increased in fetuses with neural tube defects. Whether these macrophages are present to prevent infection because of a disruption of the fetal skin or as scavenger cells to clean up neural debris is uncertain. Neutrophils are not normally identified in the AF of healthy fetuses, but are useful as a marker of AF infection. These cells are fetal in origin and appear to originate in the fetal vessels of the chorionic plate. It is interesting that meconium stained AF shows chemotactic activity for neutrophils in utero, although the meconium itself is not the likely chemotactic factor.[29] Two hematopoietic growth factors, G-CSF and macrophage colony-stimulating factor (M-CSF), are found in AF of healthy term and preterm fetuses. G-CSF is elevated in the serum of women with subclinical chorioamnionitis, in the cord blood of neonates with infection, fetal distress, premature rupture of membranes, and meconium staining of AF, and in the AF, neonatal urine and neonatal bronchoalveolar fluid of newborns after IAI. Whether G-CSF and M-CSF actually play a preventive host defense role in the AF or are just excreted by-products of the immune response during infection is not known.

There may also be nonimmune components of AF that protect the fetus from injury. For example, amniotic fluid may protect the fetal gut from the effects of platelet activating factor (PAF). PAF is a potent vasoconstrictor and has been strongly implicated in the pathophysiology of necrotizing enterocolitis in preterm infants.[30] PAF levels in human AF are low throughout gestation, but at term, PAF content undergoes an eight-fold increase with the onset of labor. PAF is elevated in AF of preterm fetuses whose mothers have failed tocolysis as well as AF of complicated pregnancies. The major PAF degrading enzymes are platelet activating factor acylhydrolase and platelet activating factor acetyl transferase; both show activity in AF, although their exact role is still unclear.[31] In addition, significant amounts of polyamines are found in AF; these have a cationic charge and may play both a nutritive and an antimicrobial role.

AF AS A DIAGNOSTIC MEDIUM

Amniocentesis has been a valuable tool in assessing fetal well-being since the 1970s. The most common evaluation of AF in the US is assessment of fetal chromosomes. Amniocentesis is commonly offered to women who will be at least 35 years of age at the time of full-term delivery or who have other risk factors for a chromosomal abnormality. As the diagnosis of aneuploidy moves into the first trimester with ultrasound assessment of nuchal translucency and more useful maternal serum markers, the use of amniocentesis will decrease with a corresponding increase in chorionic villus sampling. Amniocentesis is also offered when a previous child has a chromosomal abnormality, a parent carries a balanced chromosomal rearrangement or an autosomal recessive disorder, a mother carries an X-linked disorder, or a major structural abnormality or group of anomalies is identified on ultrasound. Assessment of AF is also helpful in the prenatal diagnosis of neural tube defects and an impressive array of inborn errors of metabolism and hematologic and genetic diseases (excellent reviews can be found in Wilson[32] and Kramer and Cohen[33]).

Evaluation of AF bilirubin level based on optical density has been an important tool to predict the severity of fetal hemolysis in red-cell alloimmunized pregnancies. Currently, the combination of amniocentesis to assess optical density, Doppler flow studies of the intra-hepatic umbilical vein and the middle cerebral artery and fetal blood sampling by cordocentesis are recommended to closely monitor the isoimmunized anemic fetus.[34] Allele-specific polymerase chain reaction of AF fetal cells can also be used to identify fetuses at risk for hemolytic disease of the newborn due to minor blood group incompatibilities.[35]

AF assessment has been studied in patients with preterm labor and/or PPROM to investigate possible IAI. AF indicators suggestive of infection include elevated levels of matrix metalloproteinase (e.g., MMP-9), interleukins (e.g., IL-6 and IL-1β), tumor necrosis factor (TNF-α), G-CSF, elevated white blood cell count, low glucose, and the presence of bacteria identified by Gram stain or culture. When preterm labor occurs with intact membranes, the rate of documented IAI is consistently lower than when preterm labor occurs with PPROM. While routine amniocentesis in preterm labor/PPROM has not been shown to be effective in decreasing perinatal mortality, there is still disagreement as to its optimum role in identification of IAI. Amniocentesis has also been helpful in prenatal diagnosis of cytomegalovirus, toxoplasma and parvovirus B-19 infection; this has become particularly relevant with the increasing use of the polymerase chain reaction allowing earlier diagnosis.

Assessment of fetal lung maturity by determination of the lecithin/sphingomyelin ratio and/or the presence of phosphatidyl glycerol in AF has become a well-accepted procedure. The assessment of lamellar body counts in AF,[36] the surfactant to albumin ratio in AF,[37] and electrical conductivity of AF[38] have

more recently been proposed as potentially superior methods for evaluation of fetal lung maturity.

A search for substances in AF that indicate fetal well-being has been ongoing since the 1980s. Changes in levels of inhibin-related proteins in both maternal serum and AF throughout pregnancy have been proposed as indicators of good fetal health. While the studies are contradictory, elevated levels of inhibin-A and activin-A may be useful markers related to fetal well-being during pre-eclampsia, trisomy 21, preterm delivery, and intrauterine growth restriction.[39] More research in this area is needed. A recent review of evaluation of AF S100B protein concentration as an early marker for brain injuries and/or brain maturation also merits further study.[40]

WHEN AF BECOMES PROBLEMATIC

Human AF may also contain substances that are potentially harmful. Perhaps the most concerning AF contaminant is meconium. There is good evidence that defecation in utero is a universal phenomenon occurring occasionally in the second trimester and frequently in the third trimester.[41] This is the likely explanation for the presence of bile pigments and enteric enzymes in AF. Meconium-stained AF occurs in about 13% of live deliveries. Most of these babies do well without associated acidosis or clinical illness. The combination of perinatal asphyxia, passage of meconium, and fetal gasping may lead to meconium aspiration syndrome (MAS), a potentially life threatening pulmonary disease caused by the combination of mechanical obstruction, inflammatory response, disruption of surfactant function, and often pulmonary hypertension (a recent review of MAS is found in Gelfand et al.[42]). MAS is uncommon in preterm infants but when present is associated with an increased risk of intraventricular hemorrhage. Meconium may also play a role in stimulating bacterial growth in the AF, perhaps by serving as an exogenous iron source. A recent study found a correlation between the presence and severity of meconium-stained AF and the rates of both chorioamnionitis and endomyometritis.[43]

AF demonstrates an irritant effect to exposed neural tissue, particularly after 34 weeks gestation. The precise identity of the irritant(s) is unclear, but there are several candidates. Tissue factor (TF), a procoagulant and initiator of disseminated intravascular coagulation, is found in high concentrations in AF at term, while TF pathway inhibitor, a natural inhibitor of TF, is found in relatively low concentrations. It is likely that TF plays a significant role in the devastating effects of AF embolism.[44] Late in pregnancy, elevated levels of activin-A and inhibin-A stimulate production of prostaglandin E2. As noted above, AF of pregnancies with premature rupture of membranes contains elevated levels of inflammatory cytokines (e.g. IL-1, IL-6, TNF-α, and interferon gamma). Whether this represents a fetal immune response or a preparatory step for the initiation of labor is not yet clear. The presence of PAF in AF with the onset of labor has been previously noted. Late in gestation AF contains vernix. While vernix contains antimicrobial substances and may be a contributor in protecting the fetus from IAI, it also has potent inflammatory properties and has been implicated as a cause of maternal antenatal peritonitis.[45] TGF-β, present late in gestation, may also play a role as a potential irritant.[46]

AF plays a major role in the gastrointestinal inflammatory changes associated with gastroschisis. An aseptic peritonitis leads to a fibrous peel, which has also been referred to as perivisceritis. The result is edema and thickening of the serosa, subserosa, and submucosa. This process has been attributed to an increase in the concentration of urea and nitrogenenous products and a decrease in the sodium and osmolality of AF that occurs at $\sim$30 weeks of gestation. Gastrointestinal waste products (i.e., bilirubin, bile acids, and meconium) have been shown to be elevated in the AF of human gastroschisis patients.[47] and in animal models are partly responsible for the perivisceritis seen in gastroschisis.[48] Amnioinfusion[49] and serial amnioexchanges[47] have both been performed in an attempt to minimize gastrointestinal inflammatory changes with preliminary results that are encouraging.

OTHER INTERESTING ASPECTS OF AF

Human AF contains factors that appear to minimize scarring.[50] It is interesting that a fetal incision made early in gestation will heal without a scar whereas one made in late gestation heals with scar formation. Two theories predominate: the first is that hyaluronic acid, which is found in high levels in AF, inhibits collagen synthesis. This hyaluronic acid-rich environment is due to a relative lack of hyaluronidase in AF and to the presence of hyaluronic acid-stimulating factor in AF. In one study looking at the effect of AF on proteases important to wound healing, human AF was shown to enhance collagenase activity, but to inhibit activities of hyaluronidase, elastase, and cathepsin.[51] The second theory is that TGF-β, which is absent from AF early in gestation but present late in gestation, plays a major role in scar formation.[46] Disagreement remains as to whether healing occurs without scar formation during early pregnancy because of a favorable fetal environment (i.e., fetal serum and AF) or because of the properties of fetal skin.

AF has been investigated as a potential way to deliver therapeutic agents to the fetus. Instillation of antibiotics, thyroxine, nutrients (i.e., dextrose, amino acids, and lipids), glucocorticoids, growth factors, surfactants, and beta-adrenergic-receptor agonists directly into the AF for delivery to the fetal circulation by either fetal swallowing or via the intramembranous route has been tried with mixed results. A 1999 National Institutes

of Health (NIH) conference on AF biology superbly summarizes this field.[52]

Human AF also contains factors that alter metabolism of opiates. Placental opioid enhancing factor has been found in placentae and AF of rats, and in placentae of humans and dolphins.[53] In cows and rats, maternal ingestion of AF enhances opioid-mediated analgesia. This effect has not been studied in humans.

Human AF has been evaluated as a source for stem cells with initial encouraging results.[54] The potential to develop a noncontroversial source of stem cells may stimulate research in this area.

UNANSWERED QUESTIONS AND FUTURE DIRECTIONS FOR AF-RELATED RESEARCH

Many research questions about AF remain unanswered. The 1999 NIH conference sponsored by the National Institute of Child Health and Development reviewed the current understanding of AF biology and important future directions for research. The conference summary called for more research in the areas of polyhydramnios, oligohydramnios, AF pressure determinations, embryonic and early fetal kidney development and function, control of lung liquid secretion, development of fetal swallowing and gastrointestinal motility, the dynamics of intramembranous absorption at the cellular and molecular level, AF pharmacokinetics and the potential therapeutic use of the amniotic space, and computer and mathematical models of AF dynamics.[52]

The functions and significance of individual growth factors in human AF remain incompletely described. It is interesting to note that some infants with esophageal atresia have malabsorption of intestinal nutrients. Other infants have a well functioning gut at birth without having swallowed significant amounts of AF. This disparity suggests that there is a redundancy of mediators that promote fetal gut growth with some effectors being swallowed in AF, while others arrive via the hematogenous route. Investigators have speculated that components of AF may protect the preterm infant against NEC or enhance intestinal recovery when NEC is in its healing stages. Components of AF that may promote these effects include glutamine,[55] arginine,[56] EGF,[57] EPO,[58] PAF-AH,[59] and LF.[60] Could harvested or synthetic AF be used as an enteral infusion in the preterm neonate at risk for or recovering from NEC? Would scarring of the gut be decreased? A recent ''simulated AF'' containing G-CSF and EPO was fed enterally to human neonates and was ''well tolerated'' at a dose of 20 ml/kg/day.[61] A follow-up study by the same investigative group showed infants tolerated simulated AF as an initial feeding when they were recovering from NEC.[62]

The skin is a major barrier to bacterial infection except in very preterm infants. Whether harvested or synthetic AF could be used to bathe and protect the not-yet-keratinized skin of the extremely preterm neonate is an appealing question. There is also much to be learned about the immunoprotective properties of AF and whether these can be enhanced to prevent IAI. There is really little information regarding how the innate host defenses of AF interact with the adaptive immune system of the mother and fetus.

Can significant amounts of AF be harvested at elective caesarean section in non-laboring women without harm to the fetus? Would this harvested AF be safe and free of infectious agents or could AF be processed (e.g., pasteurization) to render it free of infectious agents without inactivation of the desired host defense molecules? Storage and processing of AF has been investigated.[63] It is unclear whether trophic factors in AF would survive processes such as pasteurization, freezing, and storage. Given the apparent ease with which the fetus can absorb large volumes of AF in utero, would babies who are unable to tolerate regular enteral feeding (e.g. short gut, lymphatic disruption sequence, gastroschisis) be able to tolerate enteral AF infusion and thus nourish and stimulate the mucosa and minimize villous atrophy? The value of early trophic feedings in preterm infants has been well established. It is also clear that human milk is superior to premature infant formulas for these feedings. Unfortunately, breast milk is not always available. Preterm infants for whom breast milk is not available might benefit from a formula containing growth factors like those in AF and/or human milk.

Finally, as the role of VEGF in control of human AF volume becomes clear, it may be feasible to assess the role of VEGF inhibitors (e.g. bevacizumab) in the treatment of oligohydramnios and the role of VEGF receptor agonists in the treatment of polyhydramnios.

SUMMARY

AF is a wonderfully complex and unique body fluid that nourishes and protects the fetus. Just as breast milk is the optimum beverage for the newborn, AF is the ideal, germ-free bath, cushion and liquor for the fetus. Based on the significant contributions of AF to fetal and neonatal health, additional research is needed to better understand its functions and correct its disorders.

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Novel Technology to Increase Concentrations of Stem and Progenitor Cells in Marrow Aspiration

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ABSTRACT

Use of centrifuged bone marrow aspirate for regenerative medicine is a growing practice. However, such centrifugation systems require aspirating large volumes (30-240 mL) in order to obtain sufficient stem/progenitor cellularity in a large enough post-centrifugation final volume for therapeutic administration. Presented here are the results of a series of 27 marrow aspirations using **Marrow Cellution™** (www.marrowcellution.com), a bone marrow access and retrieval device designed to increase the stem/progenitor cell concentrations from the aspirate. The samples were collected under field conditions from eight separate clinicians using three different independent laboratories. The quality of the marrow aspirate was determined by performing a CFU-f test to determine the number of osteo progenitor cells.[1] Stem cells capable of forming a CFU-f are routinely found in marrow but rarely in peripheral blood. Consequently, CFU-f represents the standard test to determine the number of immature stem and progenitor cells that are present in the aspirate.[1] Previous work done by a single clinician in a controlled setting demonstrated that **Marrow Cellution™** delivered superior regenerative potential (as measured by CFU-f counts) to existing BMAC (Bone Marrow Aspiration Concentration) systems.[2] This pilot study represents true field conditions as not all clinicians followed the exact same protocol with respect to heparin rinse, orientation (posterior or anterior) and volume of aspirate taken.

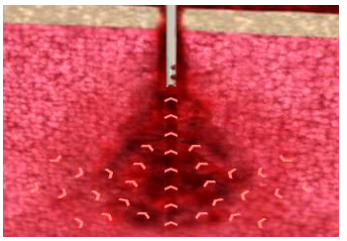
BACKGROUND

Industry often cites TNC (total nucleated cells) counts as a meaningful measure of the regenerative potential of a marrow-sourced biologic sample. TNC counts are less expensive and time-intensive to determine compared to counting osteoblast progenitor cells (as measured by CFU-f's - fibroblast-like colony-forming units). Peer reviewed literature however routinely cites CFU-f's rather than TNC's as the clinically relevant measure.[3-6] Academic studies have demonstrated a correlation between clinical outcomes and the the number of osteo-progenitor stem cells (as measured by CFU-f counts) and not TNC's.[3-6] TNC counts have limited clinical relevance because it includes nucleated red blood cells and white blood cells from peripheral blood that have reduced regenerative capability compared to marrow cells. This is especially true with biologic products that have been centrifuged because a nucleated cell from peripheral blood has the same density as a quiescent stem cell.[7-9] However, cycling progenitor stem cells have a greater density and are routinely discarded with the red cell component after centrifugation. Consequently, a centrifuge will concentrate peripheral blood nucleated cells preferentially over stem cells.

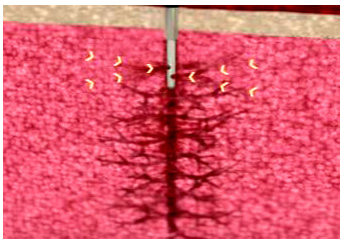
Traditional bone marrow aspiration needles were designed to aspirate 1-2 mL of marrow from a single location for diagnostic purposes.[1] When 1 mL of marrow is aspirated with a traditional needle, counts of 1451 CFU-f/mL are typical (40 x 10^6 TNC/mL).[1] When used to aspirate greater volumes that are typically required for regenerative therapies, traditional needle design results in excess peripheral blood infiltration due to basic fluid mechanics. Blood and marrow are non-Newtonian fluids and the traditional needle has a large open port at its distal end. As such it is known that peripheral blood infiltrates marrow aspirates greater than 1-2 mL when using a traditional needle due to the dramatically reduced viscosity of blood that fills the void in the medullary space that is in contact with the distal open ended lumen.

Using a traditional needle to aspirate volumes greater than 2 mL results in the initial small volume containing the most pure marrow.[10] Volume over 2 mL retrieved from a single site introduces peripheral blood into the aspiration. This peripheral blood dilutes further aspiration volume from the site and significantly reduces the stem/progenitor cell quantity of the aspiration.[1,11,12] Marrow aspiration volumes of greater than 2 mL using traditional needles typically contain only 200-300 CFU-f/mL (15-20 x 10^6 TNC/mL).[7,13] The lower viscosity of blood results in preferential aspiration of peripheral blood and a resultant precipitous decline in the stem/progenitor cells of the aspirate when larger volumes are drawn.[12,14,15] Moreover, traditional needles are technique-sensitive and not well matched to the requirement for larger aspiration volumes (60 mL) for the centrifuge to produce a final volume of 7-10 mL of autologous marrow-based therapies.[16]

Centrifuge-based systems are routinely used to overcome the limitations of lower-quality (reduced cellularity) marrow aspirations from traditional needles. These systems remove excess plasma and mature red cell count while recapturing a portion of nucleated cell content from both the marrow and the infiltrated peripheral blood components of the aspiration. These centrifuge volume reductions have become a common practice in many regenerative medicine procedures. Howev-



Traditional Needle



Marrow Cellution™

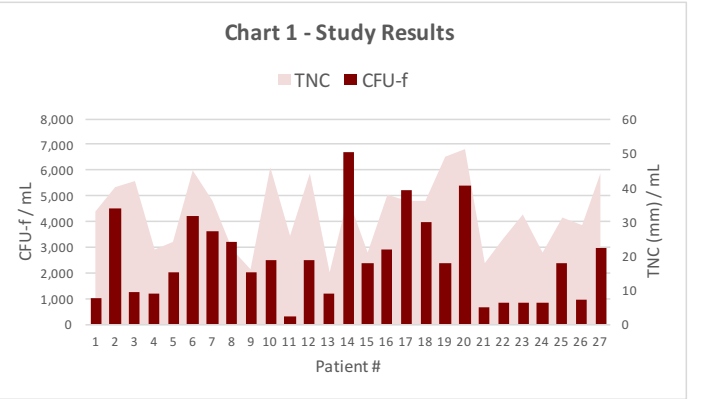
er, subsets of the nucleated cells obtained from the peripheral blood component of the aspirate may actually limit the success of procedures because nucleated cells derived from peripheral blood, rather than marrow, may stimulate an inflammatory response that can decrease the regenerative potential of the marrow-derived stem/progenitor cells.[17] More importantly, the inefficiencies of centrifuge-based systems, which have average recovery yields ranging from 32.5% to 65.2%, leads to a substantial discarding of cells in the final product.[7]

In this pilot study with **Marrow Cellution™** (Ranfac, Avon, MA), a novel bone marrow access and retrieval device co-developed by Endocellutions Corp (Marshfield, MA) and Ranfac Corp (Avon, MA), the limitations of traditional design aspiration needles and BMAC systems were substantially overcome. Flow into the aspiration system is collected laterally rather than from an open-ended cannula. This design allows for collection of marrow perpendicular to and around the channel created by the tip of the device, thus avoiding the aspiration of peripheral blood caused by the placement of the needle itself. Additionally, **Marrow Cellution™** incorporates technology to precisely reposition the retrieval system to a new location in the marrow after each 1 mL of aspiration. The effect of these two features is that multiple small volumes of high quality bone marrow aspiration are collected from a number of distributed sites within the marrow geography while also retaining clinicians' desire for a single entry point. The design enables a total volume of 8-20 mL of high quality biologic to be collected. In effect, a single puncture with **Marrow Cellution™** is functionally equivalent to repeated small aspirations (1 mL) from a number of puncture sites using traditional needles, but with substantial savings of time, effort, as well as reduced patient trauma and risk of infection.

The single-step **Marrow Cellution™** device produced the same (as counted by CFU-f's) stem/progenitor cell concentrations as a combination of traditional needles and industry-leading centrifugation systems. **Marrow Cellution™** allows the clinician to keep the product entirely on the sterile field rather than requiring the product to leave the sterile field for centrifugation. This further reduces time for the final product to be delivered to the patient (no centrifugation necessary), reduces procedural expenses, and retains all the cells and growth factors obtained in the aspiration.

Study Design

Informed consents were obtained from all patients for inclusion into the study according to ethical committee approval.



A series of 27 patients were seen by eight different clinicians and underwent marrow aspiration from the iliac crest with the **Marrow Cellution™** device using either a posterior (N=25) or anterior (N=2) orientation. A heparin rinse ranging from 500 to 2000 units/mL was used prior to aspiration. No additional heparin or anti-coagulant was used. Primary endpoints included fibroblast-like colony-forming units (CFU-f) and total nucleated cells (TNC).

Three of these patients had bilateral marrow aspiration using **Marrow Cellution™** from one iliac crest and using a traditional marrow aspiration needle the other iliac crest. The aspirations with the traditional needle were then centrifuged to produce a volume-reduced concentrate. Additionally, the aspiration volumes as well as the total volumes of the final product (aspirate for **Marrow Cellution™**; post-centrifugation for BMAC) were recorded. Descriptive statistics were used for the aspirates produced by **Marrow Cellution™**, the traditional needles, and the traditional needle/centrifuge combinations. Moreover, published literature were used to ascertain historical values for CFU-f counts from various centrifuge-based systems and compared with the aspirates produced by **Marrow Cellution™**. Finally, clinician reported estimates were gathered to determine relative preference for **Marrow Cellution™**, a traditional needle alone, or a traditional needle with centrifugation.

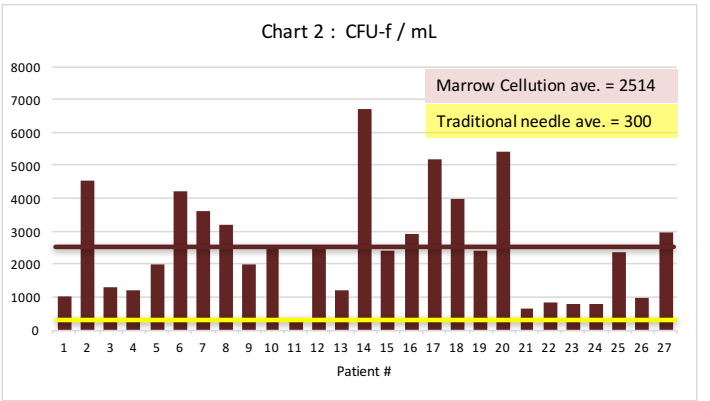
Results

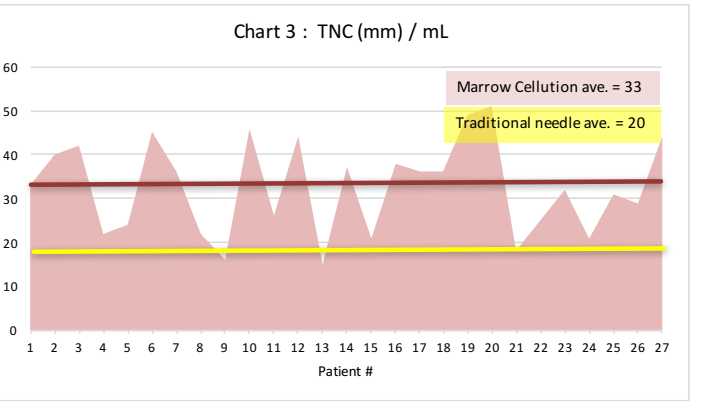
In 27 patients, 8-14 mL of marrow was collected from one iliac crest using the **Marrow Cellution™** device (aspirating from various marrow geographies from a single puncture site). Each sample was analyzed for CFU-f and TNC counts. Results for all 27 patients are depicted in Chart 1.

The average CFU-f count using **Marrow Cellution™** was 2514 (Chart 2) as compared to 200-300 CFU-f/mL using traditional needle technology.[7,13] The average TNC in the study was 33 x 10^6 TNC/mL (Chart 3) as compared to 15-20 x 10^6 TNC/mL using traditional needle technology.[7,13]

Marrow Cellution™ vs. traditional needle aspiration

In 3 patients, 8-20 mL of marrow was collected from one iliac crest using **Marrow Cellution™** (aspirating from various marrow geographies from a single puncture site); in the opposite iliac crest, 60-100 mL of marrow was collected using a





single puncture with a traditional needle. The larger volume was collected to reflect that this material is the substrate for subsequent volume reduction following centrifugation in such systems (e.g., BMAC). Two procedures used anterior entry and one used posterior. One clinician operated on two patients; and a second clinician operated on one patient. Samples of 0.5-1 mL were sent for laboratory analysis. Comparison of TNC (Chart 4) and CD34+ (Chart 5) cells were compared between **Marrow Cellution™** and the traditional needle to determine if there was a significant advantage between the two designs. With patient number 4, flow cytometry was also performed for CD34+ cells in the volume-reduced BMAC concentrate (0.140 x 10^6/mL) and was comparable to **Marrow Cellution™** (0.137 x 10^6/mL).

In three separate patients, **Marrow Cellution™** was used to collect a total of 8-10 mL of marrow aspirate. Two different clinicians performed the procedure; one surgeon used posterior access to the iliac crest, while one surgeon used anterior access. In these samples, both TNC (Chart 6) and CFU-f (Chart 7) were determined. These values were compared with published TNC and CFU-f counts from a traditional needle used to aspirate either 1 or 8 mL of marrow. The traditional needle had a significant decline in the number of stem cells aspirated per mL as the volume increased from 1 mL to 8 mL. By minimizing peripheral blood, **Marrow Cellution™** had similar number of stem cells per mL in 8 mL as the 1 mL sample from the traditional needle.

Marrow Cellution™ vs. centrifuged-based systems

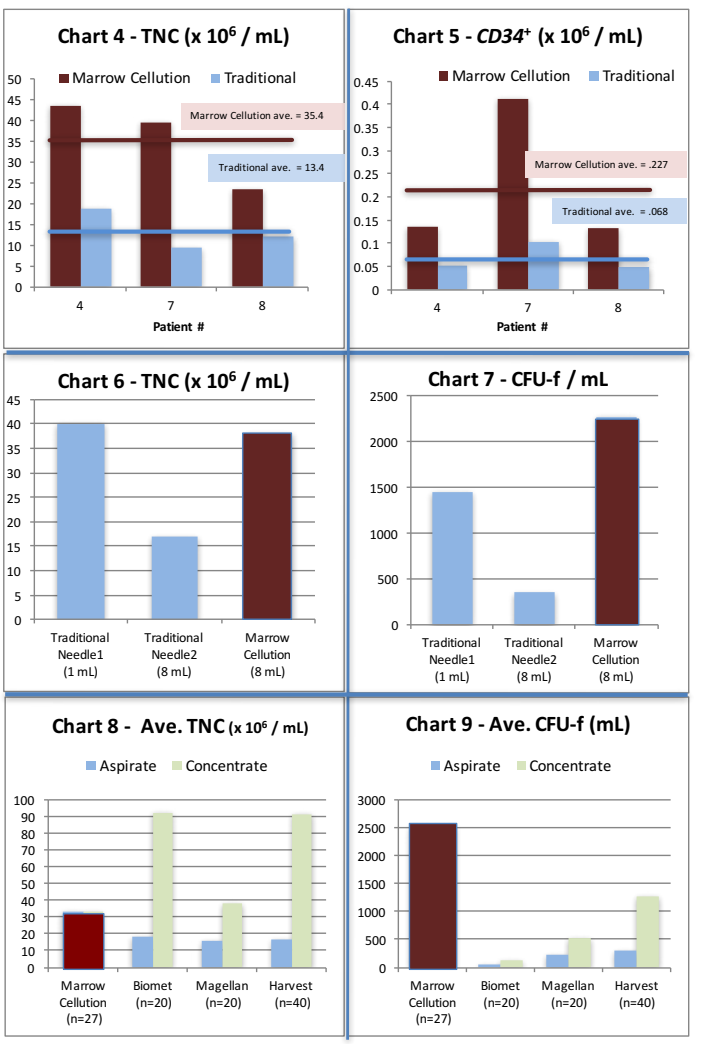
The average **Marrow Cellution™** CFU-f and TNC counts from this pilot study are compared to the average counts reported from leading centrifuged-based systems[7,16] in Charts 8 & 9.

Clinician comments on marrow aspiration technologies

Users of **Marrow Cellution™** reported that one significant advantage of the device is the ability to advance into and retreat from the marrow space in a controlled and precise manner. Along with the ability to aspirate more uniformly across the marrow geography, the **Marrow Cellution™** device produced a higher quality aspirate with the need to draw only the volume needed for the regenerative medicine treatment procedure. The clinicians also noted an improved safety profile, as the material produced does not need to leave the sterile field; in contrast, centrifuge-based technologies must leave the sterile field. Additionally, it was anticipated that substantial efficiency and cost savings would be obtained due to requiring less operating room time to prepare the marrow for use, and by eliminating the need for any specialized training beyond marrow aspiration.

Discussion

This study investigated a method to obtain equivalent stem/progenitor cells with less aspiration volume than centrifuge-based bone marrow aspirate concentrate. The **Marrow Cellution™** device provided a high quality bone marrow aspiration with reduced time and expense. The lower volume of bone marrow aspiration required can also be less traumatic on the patient and because the product remains entirely on the sterile field, risk of infection is also reduced. Our comparison



study used BMAC because of previous studies that demonstrated that BMAC produced the highest concentrations of CFU-f and CD34+ cells than other centrifuge-based systems.[7]

Conclusion

In this pilot study, the **Marrow Cellution™** device produced results suggesting that it can effectively replace aspiration of large volumes of marrow using traditional needles combined with the volume reduction of centrifuge-based systems. Traditional technologies typically discard 35-65% of cells and growth factors when reduced in centrifuge-based systems through the separation into the supernatant. These cells and growth factors are not discarded in the **Marrow Cellution™** device.

Marrow Cellution™ has a number of distinct procedural advantages: (1) the biologic produced by the device never leaves the sterile field; (2) the device requires minimal O.R. staff support and time; (3) the entire sample generated is used; (4) the device minimizes peripheral blood contamination; (5) the device requires minimal anti-coagulation; (6) the biologic does not require filtering, and (7) the design automatically repositions the aspiration cannula and aspirates from side ports across a greater geography of the marrow space so that it mimics multiple puncture sites with 1 mL aspirations. We were able to demonstrate that **Marrow Cellution™** was successful in obtaining CFU-f and TNC counts similar to what is expected from numerous insertion points along the iliac crest for multiple 1 mL-only draws; however, with **Marrow Cellution™**, only one insertion point was required.

In summary, the results documented herein from true field conditions were less than Scarpone achieved in the controlled study[2], nevertheless this pilot study clearly demonstrated superior results to previously published results from multiple centrifuged-based systems. This further suggests that the **Marrow Cellution™** device could provide even better results than BMAC alternatives as clinicians become more familiar and proficient with the device.

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