Attenuation of Cartilage Pathogenesis in Post-Traumatic Osteoarthritis (PTOA) in Mice by Blocking the Stromal Derived Factor 1 Receptor (CXCR4) With the Specific Inhibitor, AMD3100

Nathan P. Thomas,1 Pengci Li,1,2 Braden C. Fleming,1 Qian Chen,1 Xiaochun Wei,2 Pan Xiao-Hua,3 Gang Li,4 Lei Wei1,2

1Department of Orthopaedics, Warren Alpert Medical School of Brown University/RH, CORO West, Suite 402H, 1 Hoppin Street, Providence, Rhode Island 02903, 2Department of Orthopaedics, The Second Hospital of Shanxi Medical University, Shanxi Key Lab of Bone and Soft Tissue Injury Repair, Taiyuan, China, 3Department of Orthopaedic Surgery, Baohan Hospital of Southern Medical University, The 8th People’s Hospital, Shenzhen, PR China, 4Department of Orthopaedics & Traumatology, The Chinese University of Hong Kong, Hong Kong, PR China

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ABSTRACT: SDF-1 was found to infiltrate cartilage, decrease proteoglycan content, and increase MMP-13 activity after joint trauma. In this study, we tested the hypothesis that interference of the SDF-1/CXCR4 signaling pathway via AMD3100 can attenuate pathogenesis in a mouse model of PTOA. We also tested the predictive and confirmatory power of fluorescence molecular tomography (FMT) for cartilage assessment. AMD3100 was continuously delivered via mini-osmotic pumps. The extent of cartilage damage after AMD3100 or PBS treatment was assessed by histological analysis 2 months after PTOA was induced by surgical destabilization of the medial meniscus (DMM). Biochemical markers of PTOA were assessed via immunohistochemistry and in vivo fluorescence molecular tomography (FMT). Regression analysis was used to validate the predictive power of FMT measurements. Safranin-O staining revealed significant PTOA damage in the DMM/PBS mice, while the DMM/AMD3100 treated mice showed a significantly reduced response with minimal pathology. Immunohistochemistry showed that AMD3100 treatment markedly reduced typical PTOA marker expression in chondrocytes. FMT measurements showed decreased cathepsins and MMP activity in knee joints after treatment. The results demonstrate that AMD3100 treatment attenuates PTOA. AMD3100 may provide a viable and expedient option for PTOA therapy given the drug’s FDA approval and well-known safety profile. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.


Keywords: osteoarthritis; articular cartilage; SDF-1; FMT

It has been reported that aging, trauma, excessive mechanical load, and genetic factors are correlated with osteoarthritis (OA) development, while the exact mechanisms remain elusive.1–3 In particular, the treatment and prevention of post-traumatic osteoarthritis (PTOA) is essential as joint-related trauma has been highly correlated with PTOA development.4–10 Even in light of increasing research into reconstructive strategies and novel developments, joint reconstruction has not been shown to attenuate PTOA development.11–14

Chemokines and their receptors have garnered much attention given their role in immune cell function, importance for the regulation of cancer cell invasion, and role in the migration of stem cells. Specific to this study, CXCR4, is a G-protein coupled receptor that promotes activation of intracellular signaling cascades and release of MMP1 and VEGF.15,16 Originally isolated from a bone marrow stromal line, CXCR4’s ligand is the 8kDa chemokine SDF-1.17 Even though the mechanism of release remains unknown, overproduction of SDF-1 has been related to inflammatory cytokines including IL-1β and TNF-α.18,19 SDF-1 has been shown to have a variety of targets, activating primary cells by binding to the CXCR4 receptor, which in turn, stimulates chondrocyte proliferation, differentiation, and apoptosis.20,21

Recent evidence would also indicate that SDF-1/CXCR4 signaling plays an important role in PTOA progression. Looking at the distribution of SDF-1 and CXCR4 in human joints, research has shown that SDF-1 is produced in synovial membrane cells while its receptor is primarily located in articular chondrocytes.22 Briefly, SDF-1 activates the calcium, Erk and p38 MAP kinase signaling pathways, leading to the release of MMPs and other proteins.23–25 Clinical data related to synovectomies, a procedure that effectively relieves the pain associated with OA, shows a reduction in the serum SDF-1 level, decreasing intra-articular MMP release.26 The increase of SDF-1 was also found in the PTOA animal model.22,23,26,27 These results would point to the SDF-1/CXCR4 as a key pathway in regulating PTOA related cartilage degeneration.22,23,26,27 Studies have also shown SDF-1/CXCR4 signaling to play an important role in growth plate development. Through the mediation of type X collagen and MMP-13, key markers of hypertrophic chondrocyte differentiation, SDF-1/CXCR4 interaction stimulates chondrocyte hypertrophy at the chondro-osseous junction during bone formation.28

Given the alternative role of the SDF-1/CXCR4 in HIV pathways, the development of chemical agents related to pathway attenuation has been greatly accelerated. AMD3100, a specific inhibitor of the SDF-1/CXCR4 pathway, presents an ideal candidate for consideration. A bicyclam with a high specificity for the CXCR4 receptor, which demonstrates that AMD3100 treatment attenuates PTOA. AMD3100 may provide a viable and expedient option for PTOA therapy given the drug’s FDA approval and well-known safety profile. © 2015 Orthopaedic Research Society. Published by Wiley Periodicals, Inc.
corresponds to steady serum level of 0.3

In this study, we tested the hypothesis that trauma-associated, SDF-1 mediated cartilage degradation can be prevented by blocking the interaction between SDF-1 and the CXCR4 receptor on articular chondrocytes via continuous infusion of a specific inhibitor, AMD3100, in a mouse model of PTOA. We also tested the predictive and confirmatory power of in vivo fluorescence molecular tomography (FMT), a non-invasive imaging technique that can provide a quantitative measure of catabolic enzymes using specific probes.

METHODS

Animals

Twenty-eight male C57Black6/J mice (2-month-old) were obtained at 8 weeks of age (Charles River, Cambridge, MA). Mice were randomized into three groups: Group 1 (n = 8) animals underwent destabilization of the medial meniscus (DMM) on the right knee and were treated with AMD3100 via constant infusion osmotic mini-pump; Group 2 (n = 8) animals underwent DMM on the right knee and were treated with PBS via constant infusion osmotic mini-pump; and Group 3 (n = 5) animals underwent sham surgery on right knee and received empty pumps at 8 weeks. All animals were euthanized 2 months after surgery. An additional group, which underwent neither surgery nor pump implantation, was included as an additional control (n = 7). Right hind limbs were harvested immediately after euthanasia. Approval was obtained via the Institutional Animal Care and Use Committee (IACUC) at Rhode Island Hospital.

Surgery

To induce PTOA in the destabilization of the medial meniscus (DMM) subgroups, the right medial meniscotibial ligament was cut using a surgical microscope and microsurgical technique to destabilize the medial meniscus (DMM) as previously described by Glasson et al.33 Attention was paid not to injure the articular cartilage during the procedure. The right knee joints of mice in the Sham subgroups were sham-operated through the same approach without medial meniscotibial ligament injury. Post-operative animals were allowed unrestricted activity, food, and water and housed under standard conditions.

Delivery and Dosing of AMD3100

A 1.5 cm transverse skin incision was made over the dorsal thorax, and a subcutaneous pocket created via blunt dissection. The loaded Alzet osmotic minipumps (model 1004, 0.11 μL/h Alza, Palo Alto, CA) were inserted and the fascia and skin closed with 8-0 nylon, while the skin was closed with surgical staples. AMD3100 (Mozobil; Genzyme, Framingham, MA) was administered systemically. AMD3100 dosing was virtually identical to that used to successfully inhibit autoimmune joint inflammation in IFN-gamma receptor-deficient mice.34 AMD3100 was delivered at a rate of 180 μg/day, which corresponds to steady serum level of 0.3 μg/ml.35 Given the maximum duration of the Alzet osmotic pump is 4 weeks, the pumps were exchanged once. After 2 months of treatment the animals were euthanized and the knee joints removed.

Histology

The knee joints of right hind limbs were harvested and immersed in 10% (v/v) formalin for 72 h. The specimens were decalcified in 20% (v/v) EDTA solution (pH 7.2) and dissected in the sagittal plane. They were processed in a Tissue-Tek VIP 1000 tissue processor (Miles, Elkhart, IN) and embedded in a single block of Paraplast X-tra (Thermo-Fisher, Hampton, NH). The slices were cut into 6-μm sections and mounted on slides. Safranin-O staining was performed and the severity of cartilage damage was then assessed using the OARSI osteoarthritis cartilage histopathology assessment system (OOCHAS) grading system (PTOA score = Grade × Stage, total 0–24) by three independent and blinded observers, before the scores were averaged for each joint.36

Immunohistochemistry

To determine the expression of inflammatory and catabolic factors immunohistochemistry was performed. To detect the distribution of PTOA markers: MMP-13, type 2 3/4Cshort (C1, C2) and type X collagen in articular cartilage, 6-μm sections were collected on positively charged glass slides (Thermo-Fisher). Immunohistochemistry was carried out using the DAB Histostain-SP Kit (Zymed-Invitrogen, Carlsbad, CA). Sections were prepared via standard methods. The sections were incubated with specific antibodies against MMP-13 (Santa Cruz, Santa Cruz, CA), type 2 3/4Cshort (C1, C2), which detects fragments of both type I and type II collagen produced by the action of collagenase (IBEX, Montreal, Quebec), and type X collagen (Santa Cruz) respectively at 4°C overnight. Following staining, slides were qualitatively analyzed for the expression of markers. Photography was performed with a Nikon E800 microscope (Nikon, Melville, NY).

Fluorescence Molecular Tomography (FMT)

Using in vivo FMT imaging methods, real-time information was gained about biological processes using probes and deep tissue imaging.37–39 ProSense and MMPSense, both protease-activated near-infrared (NIR) fluorescence imaging probes, detect cathepsin and MMP activity respectively. We used FMT imaging at three months to confirm that AMD3100 treatment reduced the presence of inflammatory reactions associated with PTOA pathology. We also used FMT imaging to confirm histological scores and immunohistochemical data. Similar to the methodology used by Zhou et al.40 at 2 months, mice were injected with single dose of ProSense 750EX and MMPSense 680 fluorescent agents (PerkinElmer, Waltham, MA) 24 h before imaging. The animal was anesthetized using an intraperitoneal injection of ketamine (75 mg/kg) and medetomidine (1 mg/kg), mice were placed in an upright position in the imaging chamber and then imaged with the FMT system (ViSen, Waltham, MA). A NIR laser diode emitting continuous wave radiation at wavelengths of 670 or 746 nm transilluminated the lower body of animal from posterior to anterior, and both excitation and emission signals were detected by a charge-coupled device (CCD) camera and appropriate band pass filters. ProSense detects cathepsin B, L, S, and plasmin. MMPSense detects MMP-2, 3, 9, and 13 activities. The DMM/PBS group (N = 5), DMM/AMD3100 group (N = 6), and Sham group (N = 6), were
Concentrations of the probes in the knee joint were determined using Region of Interest (ROI) analysis, by restricting the area of measurement to the mid-femur to mid-tibia in order to isolate the joint space.

**Statistical Analysis**

The OOCHAS score in different groups were analyzed by one-way ANOVA with multiple pair-wise comparisons made by the Student–Newman–Keuls method (three comparisons or more) at a rejection level of 5% unless otherwise noted. Mixed linear models were used to compare cartilage inflammation measurements via in vivo FMT measurements. Residual estimates of maximum likelihood were used to fit the models to provide unbiased estimates for missing data due to the small sample size of FMT scans. Post hoc paired comparisons among the three experimental groups were carried out with orthogonal contrasts using the Spearman’s rank test to maintain alpha at 0.05. Adjusted p values are reported to account for multiple comparisons. All data are presented as means and p values of the (operative limb treated with AMD3100-control sham limb). The relationships between the Mankin score versus the FMT quantifications of inflammation were assessed with regression analysis. All statistical analyses were done in STATA SE 12.1 (StataCorp, College Station, TX).

**RESULTS**

**Histology**

Cartilage histology revealed a safranin-O positive articular cartilage surface in the DMM/AMD3100

![Image](image_url)

**Figure 1.** AMD3100 treatment prevents OA cartilage damage. Safranin-O staining shows the changes in proteoglycan and cartilage structure in representative (median OOCHAS scoring) joints. (A) DMM/PBS, (B) DMM/AMD3100, (C) Sham control, (D) Sham—No surgery control.
treatment group with a preserved and intact cartilage surface similar to the Sham control (Fig. 1). As expected, the DMM/PBS group showed an extensive reduction in proteoglycan content and severe cartilage damage with matrix erosion at the surface.

Using the OOCHAS score, the extent of OA damage was quantified by blinded observers. Severe OA damage was quantified in the DMM/PBS group, while the DMM/AMD3100 treatment group displayed significantly lower scores ($p < 0.001$) not significantly different from either the sham or No-surgery control groups ($p = 0.704, 0.169$, respectively) (Fig. 2). Related to the safranin-O staining, and in line with the literature, the OOCHAS scores of the sham with pump and no surgery groups were not significantly different from zero ($p < 0.001$) as they showed minimal PTOA damage (Fig. 1).

**Immunohistochemistry**

In the DMM/PBS group, expression of MMP-13 was elevated compared to the DMM/AMD3100 and Sham groups (Fig. 3). Expression of Col 2 3/4 short (C1-2C) and Col X were markedly reduced in the AMD3100 treatment and sham groups, where less PTOA damage was observed, compared to the PBS control, where extensive PTOA damage was observed. Staining was uniform across cartilage surfaces.

**Fluorescence Molecular Tomography (FMT)**

The MMPSense signal was significantly reduced from 0.55 pmols in the DMM/PBS group to 0.29 pmols in the DMM/AMD3100 group ($p < 0.05$). Similarly, a significant positive correlation was ($p < 0.001; N = 17$) was found between the MMPSense signals and the OOCHAS scores across all the study groups ($r^2 = 0.899$). Similarly, AMD3100 treatment significantly reduced the ProSense signal from 1.42 pmols in the DMM/PBS group to 0.61 pmols in the DMM/AMD3100 group ($p < 0.05$; Fig. 4). A significant positive correlation was ($p < 0.001; N = 17$) also found between the ProSense signals and the OOCHAS scores ($r^2 = 0.923$; Fig. 5).

![Figure 2](image2.png) **Figure 2.** Blockage of SDF-1/CXCR4 by AMD3100 attenuated OA. OOCHAS scores are shown. Mean ± SD. DMM/PBS, $N = 8$. DMM/AMD3100, $N = 8$. Sham—pump only, $N = 5$. Sham—no surgery, $N = 7$. There were no significant differences between the two control groups, but there was as significant difference ($p < 0.001$) between the DMM/PBS group and the DMM/AMD3100 group.

![Figure 3](image3.png) **Figure 3.** MMP-13, Col X, and Col 2 3/4 short expression are reduced in AMD3100 treated mice. Immunohistochemical staining was done on cartilage, and representative images are presented. Positive signal is indicated by brown/red staining.
DISCUSSION

Our data suggests that PTOA associated articular cartilage pathogenesis can be prevented by continuous infusion of a specific CXCR4 antagonist, AMD3100, further confirming the role of the SDF-1/CXCR4 pathway in PTOA development. This study also highlights the predictive power of in vivo fluorescence molecular tomography for estimation of cartilage degradation.

While extensive cartilage degeneration was observed in the control (DMM/PBS), minimal damage was observed in the treatment group (DMM/AMD3100) indicating that AMD3100 effectively prevented PTOA associated articular cartilage structural damage (Fig. 1). The structural damage detected using histology was also supported by both the biochemical and in vivo FMT inflammation data, which demonstrate that AMD3100 treatment significantly reduces inflammatory signals associated with the production of proteases after DMM, associated with decreased OA pathology in the same treated mice at both a gross and biochemical level. It is well known that increased

Figure 4. AMD3100 treatment reduces inflammation signals determined by FMT. Cathepsin and MMP activity are decreased in AMD3100 treated joints at 3 months after DMM surgery. (A) Representative MMPSense and ProSense images and (B) summary data means are shown. DMM/PBS, N = 5; DMM/AMD3100, N = 6; Sham, N = 6.
MMP-13 expression leads to matrix degradation and knockdown of MMP-13 reduces OA associated cartilage damage in mice.\(^4^1\) Downregulation of C1-2C confirms reduced cleavage of both type II and type I collagen by MMPs in both bone and cartilage, respectively.\(^4^2\) Work by Shen et al.,\(^4^3\) which examined the effects of hMeSPC injection on articular protection, has shown the importance of SDF-1 signaling on meniscus repair in PTOA. Although these two studies focus on the same pathway, the present work tests the natural role of the SDF-1/CXCR4 pathway in PTOA progression, rather than the enhancing effect of SDF-1 treatment on hMeSPC migration and morphology for meniscus repair.

We have also demonstrated that in vivo FMT measurements are not only highly correlated with morphological and immunohistochemical data, but that, FMT measurements have the potential to predict PTOA cartilage development (Fig. 2). FMT has previously been used in a variety of fields as a non-invasive tool to measure inflammatory diseases.\(^3^7-3^9,4^4\) FMT represents an optimal technique for non-invasive, longitudinal inflammation measurements and continuous monitoring of disease progression, as measurements have been used to sensitively predict disease development in rheumatoid arthritis.\(^4^4\) This study has shown both ProSense and MMPSense probes may be correlated with morphological results in PTOA pathogenesis in the murine model.

For patients with a known risk of developing PTOA, this study is particularly relevant. Given a reported window of PTOA onset and disease progression, successful AMD3100 dosing may have the ability to attenuate the biochemical progression of PTOA-associated articular cartilage degradation following reconstructive or corrective surgery. Further research will be required to evaluate the long-term efficiency and pharmacokinetic profile of AMD3100, but the present results are encouraging. Additional research will also be required to evaluate the potential of AMD3100 to attenuate global PTOA progression after the initial onset of disease.

A potential limitation to this study is that surgical DMM 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 PTOA. Recognizing the role of SDF-1 signaling in the recruitment of reparative cells, it will be important to demonstrate that use of this drug neither increases the time for general wound healing nor restoration of subchondral cystic changes that often occur in association with traumatic joint injury. Research by Shen et al. demonstrated that AMD3100, in clinical situations involving surgical repair of the meniscus, may be contraindicated as the SDF-1 axis has been implicated in meniscus repair. Nonetheless, the animal ACLT model has been frequently used to study PTOA, and mimics human OA both macroscopically and biochemically.\(^3^3\) Minimizing joint innate immunity inflammation until ACL reconstruction is performed may be an important preventative measure against the long-term development of PTOA.

In summary, the results of this study demonstrate that pharmacologic inhibition of SDF-1 signaling within the joint may provide protection against cartilage destruction in a mouse model of PTOA and highlights the potential of AMD3100, an FDA approved drug, as an effective attenuator of the SDF-1/CXCR4 pathway. Future clinical studies should explore orthopedic opportunities for pharmacologic inhibition of the SDF-1/CXCR4 signaling pathway, given AMD3100 provides as a promising agent for therapeutic use given its high specificity and well known safety profile in humans.\(^3^0,4^5\)

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REFERENCES


25. Chernock RD, Cherla RP, Ganju RK. SHP2 and cbl participate in alpha-chemokine receptor CXCR4-mediated


