An aggrecan fragment drives osteoarthritis pain through Toll-like receptor 2

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Abstract

Pain is the predominant symptom of osteoarthritis, but the connection between joint damage and the genesis of pain is not well understood. Loss of articular cartilage is a hallmark of osteoarthritis, and it occurs through enzymatic degradation of aggrecan by ADAMTS-4/5-mediated cleavage in the interglobular domain (E373-374A). Further cleavage by MMPs (N341-342F) releases a 32-amino-acid aggrecan fragment (32-mer). We investigated the role of this 32-mer in driving joint pain. We demonstrated that the 32-mer excites dorsal root ganglion (DRG) nociceptive neurons, both in culture and in intact explants. Treatment of cultured sensory neurons with the 32-mer induced them to express the pro-algesic chemokine, MCP-1/CCL2. These effects were mediated through Toll-like receptor (TLR)2, which we demonstrated was expressed by nociceptive neurons. In addition, intraarticular injection of the 32-mer provoked knee hyperalgesia in wild-type but not Tlr2 null mice. Blocking the production or action of the 32-mer in transgenic mice prevented the development of knee hyperalgesia in a murine model of osteoarthritis. These findings suggest that the aggrecan 32-mer fragment directly activates TLR2 on joint nociceptors and is an important mediator of the development of osteoarthritis-associated joint pain.
Introduction

An early event in the pathogenesis of osteoarthritis is enzymatic cleavage of the major cartilage proteoglycan, aggrecan, in the interglobular domain (E$^{373\downarrow374}$A) (1-3) by ‘a disintegrin and metalloproteinase with thrombospondin motif 4’ (ADAMTS-4) or ADAMTS-5 (4, 5). This results in loss of the bulk of the aggrecan molecule from the articular cartilage, and therefore, this cleavage step is critical for the development of osteoarthritis (6, 7). Accordingly, many pharmaceutical programs over the last two decades have focused on developing disease-modifying osteoarthritis drugs targeting ADAMTS-4 and ADAMTS-5 (8-10).

Once aggrecan is cleaved in the interglobular domain by ADAMTS-4/ADAMTS-5, the remaining 70-kDa N-terminal aggrecan fragment is retained in the cartilage matrix and is subsequently cleaved by matrix metalloproteinases (MMPs) at N$^{341\downarrow342}$F, releasing a 32-amino acid fragment (32-mer, $^{342}$F-E$^{373}$) (11). This 32-mer has been identified in synovial fluid from osteoarthritis patients (12), and we have demonstrated that this fragment can promote pro-inflammatory signaling in chondrocytes, synovial fibroblasts and peritoneal macrophages, through the activation of TLR2 (13). We recently reported that nociceptors can directly respond to Damage-Associated Molecular Patterns (DAMPs) present in the osteoarthritis joint, such as S100A8 and α2-macroglobulin, by activating TLR4 receptors expressed by joint nociceptors (14). Thus, it is likely that nociceptors might also sense and respond to other DAMPs, particularly those released from the cartilage extracellular matrix as a result of ongoing degradation.
Results and Discussion

We sought to determine whether the 32-mer aggrecan fragment produces pro-algesic effects. We first examined its ability to directly excite DRG neurons by examining its effects on intracellular calcium mobilization, \([\text{Ca}^{2+}]_i\). Cultured DRG neurons from wild-type mice rapidly responded to 32-mer peptide as indicated by increased \([\text{Ca}^{2+}]_i\) in 23% of neurons, suggesting that a subpopulation of DRG neurons express excitatory receptors for this protein fragment (Table 1, Supplementary Figure 1A). Scrambled control peptide induced responses in 6% of neurons (Table 1). All 32-mer responses were seen in small-to-medium-diameter neurons, consistent with the size of nociceptors (Supplementary Figure 1B). In addition, the majority of neurons that responded to the 32-mer peptide also responded to capsaicin (55/71 neurons = 77%), demonstrating that a subset of TRPV1 (transient receptor potential cation channel subfamily V member 1)-expressing nociceptors can respond to 32-mer. The synthetic TLR2 ligand, Pam3CSK4, also induced responses in 12% of neurons (9/76) (Supplementary Figure 1C), primarily in small-to-medium diameter, capsaicin-sensitive neurons. Finally, in DRG cultures from Tlr2 null mice, only 5% of neurons responded to 32-mer, suggesting that excitation is dependent on this signaling pathway (Table 1). In order to show that 32-mer responses are not an artifact of cell culture, calcium imaging was also performed using intact DRG from Pirt-GCaMP3 expressing mice (14, 15). In three intact DRG explants, 6% of capsaicin-sensitive neurons (12/204) responded to 32-mer peptide, while scrambled peptide and vehicle solution each elicited responses in only 2% of capsaicin-sensitive neurons (4/204) (p=0.037) (Supplementary Figure 1D), supporting the cell culture findings.

In order to examine the potential pro-algesic effects of 32-mer signaling in DRG neurons, we stimulated cultured DRG cells from wild-type mice with 32-mer peptide and measured release of the chemokine, monocyte chemoattractant protein-1 (MCP-1/ CCL2) into culture medium. We previously found that MCP-1 is upregulated by DRG neurons in experimental osteoarthritis induced by surgical destabilization of the medial meniscus (DMM) and acts as a key mediator of osteoarthritis pain (16). Overnight incubation of cultured DRG cells with 3 or 30 μM 32-mer peptide resulted in significant upregulation in MCP-1 production compared with unstimulated cells (3.1-fold (3 μM) and 3.5-fold (30 μM), p<0.0001) (Figure 1A). The highest concentration of scrambled peptide (30 μM) did not induce MCP-1 production (0.8-fold, p>0.9999 vs. unstimulated). Since this portion of the
aggrecan molecule can be modified by keratan sulfate chains (17-20), we next tested whether the glycosylated native aggrecan 32-mer fragment can also stimulate DRG cells using native 32-mer purified from porcine cartilage. We found that native 32-mer stimulated DRG cells to produce elevated levels of MCP-1 compared with unstimulated cells (3.1-fold (10 μM), p<0.01) (Figure 1B).

Which receptors mediate the effects of the 32-mer on DRG nociceptors? In order to answer this question, we prepared DRG cultures from Tlr2 null or Tlr4 null mice. Stimulation with 32-mer peptide (3 μM) produced increased MCP-1 in Tlr4 null (3.3-fold, p<0.01) (Figure 1C), but not in Tlr2 null DRG cultures (0.9-fold, p=0.5) (Figure 1D), suggesting that these effects are mediated through the activation of TLR2. Wild-type DRG cells also responded to Pam3CSK4 (10 μM, 7.7-fold, p<0.0001) (Figure 1E) while Tlr2 null DRG cells did not (Figure 1F), providing additional evidence that TLR2 signaling can lead to increased MCP-1 expression by these neurons. TLR2 is known to be expressed in a variety of cells as functional heterodimers with either TLR1 or TLR6, but no reports have demonstrated expression by DRG neurons (21). Therefore, we immunostained DRG sections from wild-type naïve mice and found that 17±2% of all DRG neurons stained positive for both TLR1 and TLR2, and 12±2% of all DRG neurons stained positive for both TLR6 and TLR2 (Supplementary Figure 2). In addition, naïve Tlr2-lacZ+/- reporter mice also demonstrated TLR2 expression in DRG neurons (Supplementary Figure 3).

Next, we aimed to determine whether the 32-mer has the potential for generating pain in vivo. We first injected Pam3CSK4 into the knee cavity of naïve wild-type mice, which caused knee hyperalgesia in a dose-dependent fashion (Figure 2A). An intra-articular injection of lidocaine, administered once peak Pam3CSK4-induced knee hyperalgesia was established, reversed knee hyperalgesia, indicating a direct effect of peripheral nerves in mediating the observed hyperalgesia (Figure 2B). Tlr2 null mice did not develop knee hyperalgesia following injection of Pam3CSK4 into the knee cavity, indicating that hyperalgesia is indeed induced through the TLR2 pathway (Figure 2C). Next, we directly tested the effects of the 32-mer by injecting either the 32-mer or scrambled peptides into the knee cavity of naïve mice. We observed that the 32-mer but not the scrambled peptide induced acute knee hyperalgesia in wild-type (Figure 2D) but not Tlr2 null mice (Figure 2E).

These data indicate that activation of TLR2 receptors by the 32-mer can elicit acute pain. In order to determine whether this process contributes to pain in active osteoarthritis,
we explored the role of the 32-mer in generating knee hyperalgesia in an experimental model of osteoarthritis induced by DMM surgery. We have previously validated the DMM model as a suitable preclinical model that captures the long-term progression of osteoarthritis and associated pain (22, 23). We performed DMM surgery in wild-type, “Chloe”, and Tlr2 null mice. Chloe mice are a transgenic line in which the matrix metalloprotease-cleavage site (N\(^{341}\)↓\(^{342}\)F) in the aggrecan interglobular domain is eliminated by amino acid changes, thus preventing production of the 32-mer fragment (24). However, since the aggrecanase-cleavage site (E\(^{373}\)↓\(^{374}\)A) in the interglobular domain is not modified, Chloe mice still develop osteoarthritis after DMM surgery (6). In this study, Chloe mice developed more cartilage degeneration and larger osteophytes than wild-type mice four and sixteen weeks after surgery (Supplementary Figure 4A,B). Subchondral bone sclerosis was also increased in Chloe mice compared with wild-type mice 4 weeks after DMM (Supplementary Figure 3C). Similar synovial changes were seen in both wild-type and Chloe mice throughout the 16 weeks (Supplementary Figure 4D). Wild-type and Tlr2 null mice developed similar joint damage by 16 weeks after DMM (Supplementary Figure 4).

Similar to our previous results (25), DMM surgery caused pronounced knee hyperalgesia in wild-type mice by 2 weeks after surgery compared with sham mice, and hyperalgesia slowly resolved through 16 weeks (Figure 3A). Four weeks after DMM, intra-articular injection of lidocaine reversed knee hyperalgesia (Figure 3B), indicating that it is a locally-generated pain-related behavior. We have also previously demonstrated that knee hyperalgesia is reversed by systemic injection of morphine (25). In contrast, Chloe mice were protected from developing knee hyperalgesia until 12 weeks after DMM surgery compared with wild-type mice (Figure 3C), suggesting that the 32-mer fragment mediates the development of knee hyperalgesia. 32-mer peptide injected into the knee joint of naïve Chloe mice induced knee hyperalgesia similar to wild-type naïve mice (Figure 3D, Figure 2D), confirming that these mice retain the ability to respond to the 32-mer. Tlr2 null mice were also protected from knee hyperalgesia up to 16 weeks after DMM surgery (Figure 3D), supporting the hypothesis that 32-mer signaling through TLR2 activation plays a key role in mediating early phase knee hyperalgesia in this model.

Finally, in order to test whether the 32-mer plays a role in mediating other DMM-associated chronic pain behaviors, we tested whether mechanical allodynia of the ipsilateral hindpaw was reduced after blocking the creation or action of the 32-mer. We found that
wild-type, Chloe, and Tlr2 null mice developed similar patterns of mechanical allodynia in the ipsilateral hindpaw through 16 weeks after DMM (Supplementary Figure 5), suggesting that the primary action of the 32-mer aggrecan fragment on pain is locally within the knee joint.

Toll-like receptor signaling has been implicated in a variety of neuro-immune processes (21, 26). TLR2 has been shown to be expressed by satellite glial cells in the DRG, and by microglia, astrocytes, and neurons within the central nervous system (21, 27). In nerve injury models, Tlr2 null mice are partially protected from developing mechanical allodynia (28), microglial activation in the spinal cord (28), and macrophage infiltration into the DRG (29). Here, we demonstrate for the first time that TLR2 can be co-expressed with TLR1 and TLR6 by DRG sensory neurons, joining the list of other TLRs (TLR3, TLR4, TLR5, TLR7, and TLR9) that have also been shown to be expressed by DRG sensory neurons (14, 21, 30).

A select group of extracellular matrix molecules, including low molecular weight hyaluronan, fibronectin-EDA, fragments of fibronectin, tenascin C, and biglycan have been shown to act as DAMPs, signaling through TLR2 and TLR4 on non-neuronal cells (31). Recently, we have added the 32-mer fragment of aggrecan to this list, by demonstrating that it can upregulate catabolic signaling through TLR2 expressed by chondrocytes, synovial fibroblasts, and macrophages (13). Chondroitin-sulfate proteoglycans, including aggrecan, have been shown to play a role in guiding the development and growth of the sensory nervous system as well as regeneration following injury (32). Here, we show for the first time that an extracellular matrix fragment, specifically, the 32-mer aggrecan fragment, can signal through TLR2 on sensory neurons. It will be interesting to investigate the effects of other extracellular matrix fragments on sensory neurons in future work.

In conclusion, we have demonstrated that activation of TLR2 receptors expressed by nociceptors can promote pro-algesic signaling, particularly the production of the chemokine MCP-1 by these neurons, which we have previously identified as an important mediator of osteoarthritis pain (23). Our findings suggest that the 32-mer fragment of aggrecan directly activates TLR2 expressed by nociceptors within the knee joint, driving the development of osteoarthritis-associated knee hyperalgesia. These results provide new evidence indicating that molecules derived from the process of ongoing cartilage degradation can directly act
upon nociceptors thereby integrating osteoarthritis pain with other aspects of joint degeneration.
Methods

Additional methods are provided in the Supplemental Methods.

Statistics. For calcium imaging experiments, Chi-squared tests were used to compare the number of responses. For MCP-1 stimulation experiments, one-way ANOVA with Bonferroni post-tests or unpaired t-tests assuming equal variances were used to compare the groups of interest. For knee hyperalgesia following intra-articular injection time courses, a repeated measures two-way ANOVA with Bonferroni post-tests was used to compare vehicle vs. drug at each time point. For knee hyperalgesia time courses in mice following DMM surgery, a two-way ANOVA with Bonferroni post-tests was used to compare responses at each time point. For mechanical allodynia time courses, one-way ANOVA with Bonferroni post-tests was used to compare each time point to time 0. For knee histopathology, data were analyzed using two-way ANOVA with Bonferroni post-tests or Mann-Whitney test. All analyses were carried out using GraphPad Prism version 6.07 for Windows (GraphPad Software, San Diego, CA). Results are presented as mean ± SEM or median ± IQR, as indicated.

Study approval. All experiments were approved by the Rush University Institutional Animal Care and Use Committee.
Author Contributions

REM, AMM, and RJM were responsible for overall study design. REM performed calcium imaging and MCP-1 experiments, collected knee joints for histopathology, and performed statistical analyses. SI performed DMM surgery and assessed knee hyperalgesia and mechanical allodynia, and other in vivo techniques. PBT performed the immunohistochemistry. SBG, KL and AJF supplied the synthetic 32-mer peptide, native 32-mer fragment, and Chloe mice. AJF also provided input on overall experimental design and analysis of the data. The manuscript was written by REM, RJM, and AMM. All authors were involved in the design of the parts of the study they executed, discussed the design and results, commented on the manuscript and approved the final version.
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References


Figure Legends

Figure 1. The 32-mer upregulates MCP-1 protein production through TLR2 in cultured DRG cells. A) Wild-type DRG cells were stimulated with 0-30 μM synthetic 32-mer or scrambled peptide. Repeated 0 vs 3 μM synthetic 32-mer in 4 independent cultures. B) Wild-type DRG cells were stimulated with 10 μM synthetic or native porcine 32-mer in the presence of 30 μg/mL polymyxin-B. Representative of two independent experiments. C) Tlr4−/− DRG cells were stimulated with 0 vs 3 μM synthetic 32-mer. Representative of two independent experiments. D) Tlr2−/− DRG cells were stimulated with 0 vs 3 μM synthetic 32-mer. Representative of three independent experiments. E) Wild-type DRG cells were stimulated with 0 vs 1 μg/mL synthetic TLR2 ligand Pam3CSK4. Representative of three independent experiments. F) Tlr2−/− DRG cells were stimulated with 0 vs 1 μg/mL synthetic TLR2 ligand Pam3CSK4. Representative of two independent experiments. **p<0.01, ***p<0.001, ****p<0.0001; mean±SEM.

Figure 2. Intra-articular injection of synthetic 32-mer of Pam3CSK4 induces knee hyperalgesia in naïve mice. A) Intra-articular injection of vehicle, 1 or 3 μg synthetic TLR2 ligand Pam3CSK4 in wild-type mice. n=4 mice/treatment. B) Intra-articular injection of 3 μg synthetic TLR2 ligand Pam3CSK4 in wild-type mice followed by a second intra-articular injection of vehicle or lidocaine (20 mg/kg). n=5 mice/treatment. C) Intra-articular injection of vehicle or 3 μg synthetic TLR2 ligand Pam3CSK4 in Tlr2−/− mice. n=4/vehicle; n=6/Pam3CSK4. D) Intra-articular injection of 10.5 μg synthetic 32-mer or scrambled peptide in wild-type mice. n=4/scrambled; n=5/32-mer. E) Intra-articular injection of 10.5 μg synthetic 32-mer peptide in wild-type or Tlr2−/− mice. n=5/strain. ***p<0.001, ****p<0.0001; mean±SEM.

Figure 3. Blocking the production or action of the 32-mer protects against early knee hyperalgesia following DMM surgery. A) Time course of knee hyperalgesia in wild-type mice following DMM surgery. n=6-8 mice/group. B) Intra-articular injection of vehicle or lidocaine (20 mg/kg) four weeks after DMM surgery. n=4 mice/treatment. C) Time course of knee hyperalgesia in wild-type (n=5) vs. Chloe (n=4 mice) after DMM surgery. D) Intra-articular injection of 10.5 μg synthetic 32-mer or scrambled peptide in naive Chloe mice. n=4/group. E) Time course of knee hyperalgesia in Tlr2−/− mice following sham or DMM surgery. n=5 mice/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; mean±SEM.
Table 1. Intracellular calcium responses in cultured DRG neurons

<table>
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<tr>
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<th># neuronal responses / total # neurons</th>
<th>% responding</th>
<th>p-value (Chi-square test)</th>
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<tr>
<td>WT: 32-mer peptide (3 µM)</td>
<td>71/309</td>
<td>23%</td>
<td>p&lt;0.0001</td>
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<tr>
<td>WT: Scrambled peptide (3 µM)</td>
<td>18/299</td>
<td>6%</td>
<td></td>
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<tr>
<td>Tlr2 null: 32-mer peptide (10 µM)</td>
<td>3/57</td>
<td>5%</td>
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C) Time course of knee hyperalgesia in wild-type (n=5) vs. Chloe (n=4 mice) after DMM surgery. 

D) Intra-articular injection of 10.5 μg synthetic 32-mer or scrambled peptide in naïve Chloe mice. n=4/group. 

E) Time course of knee hyperalgesia in Tlr2-/- mice following sham or DMM surgery. n=5 mice/group. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; mean±SEM.