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Protease-activated receptor 2: a novel pathogenic pathway in a murine model of osteoarthritis

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ABSTRACT

Objective Osteoarthritis is a global clinical challenge for which no effective disease-modifying agents currently exist. This study identified protease-activated receptor 2 (PAR-2) as a novel pathogenic mechanism and potential therapeutic target in osteoarthritis.

Methods Experimental osteoarthritis was induced in wild-type and PAR-2-deficient mice by sectioning the medial meniscotibial ligament (MMTL), leading to the development of a mild arthropathy. Cartilage degradation and increased subchondral bone formation were assessed as indicators of osteoarthritis pathology.

Results Four weeks following MMTL section, cartilage erosion and increased subchondral bone formation was evident in wild-type mice but was substantially reduced in PAR-2-deficient mice. Crucially, the therapeutic inhibition of PAR-2 in wild-type mice, using either a PAR-2 antagonist or a monoclonal antibody targeting the protease cleavage site of PAR-2, was also equally effective at reducing osteoarthritis progression *in vivo*. PAR-2 was upregulated in chondrocytes of wild-type but not sham-operated mice. Wild-type mice showed further joint degradation 8 weeks after the induction of osteoarthritis, but PAR-2-deficient mice were still protected.

Conclusions The substantial protection from pathology afforded by PAR-2 deficiency following the induction of osteoarthritis provides proof of concept that PAR-2 plays a key role in osteoarthritis and suggests this receptor as a potential therapeutic target.

Osteoarthritis is a chronic disabling condition currently affecting millions globally,¹ with radiological evidence of osteoarthritis in approximately 80% of the population aged over 65 years.² Osteoarthritis is characterised by cartilage degradation and increased subchondral bone formation (osteosclerosis). Despite extensive pathophysiological investigations, clinical management has not altered significantly and comprises the administration of analgesics and non-steroidal anti-inflammatory agents and recourse upon joint failure to arthroplasty. No unifying pathogenetic model exists—suggested hypotheses encompass primary cartilage metabolic dysregulation, enthesial disease together with biomechanical dysregulation. Thus far, no critical checkpoint pathway has been identified that is essential for disease progression and which might by corollary represent a valid, disease-modifying osteoarthritis therapeutic target. Protease-activated receptor 2 (PAR-2) is a G-protein coupled receptor whose ‘tethered’ ligand is activated by serine proteases.³ PAR-2 is present in chondrocytes in cartilage from osteoarthritis patients,⁴ and following its activation, matrix metalloproteases (MMP)

are generated.⁵ However, these previous observations are associative and do not establish the role of PAR-2 in the pathogenesis of osteoarthritis. In this study we sought direct evidence of a causal relationship between PAR-2 expression and cartilage and bone pathology in a murine model of osteoarthritis.

METHODS

Animals

Experiments were performed on adult wild-type (PAR-2^{+/+}) C57BL/6J mice (body weight 25–30 g), housed in standard cages, with food and water freely available and maintained in a thermoneutral environment. PAR-2-deficient (PAR-2^{-/-}) mice were genetically modified⁶ and maintained under the same conditions. All procedures were performed in accordance with UK Home Office regulations.

Induction of experimental osteoarthritis

Two previously described models of joint instability were employed.⁷ The principal model used involved destabilisation of the medial meniscus by section of the medial meniscotibial ligament (MMTL), which results in the development of a mild arthropathy resembling human osteoarthritis.⁷ A more severe model, involving anterior cruciate ligament (ACL) transection, was induced in a smaller group of mice.⁷ All surgical procedures were performed by the same investigator who was blinded to genotype or treatment. Following surgery, animals were left for 4 or 8 weeks and thereafter, knee joints were harvested for subsequent histological analysis. Knee joint diameter was measured weekly for a month following MMTL surgery in a small cohort of mice, but no significant difference occurred over time ($p=0.6$, one-way analysis of variance (ANOVA), $n=6$). As similar results were obtained with the ACL section model, no further such measurements were performed.

Assessment of cartilage damage

Histological analysis of progression and severity of cartilage damage was undertaken on harvested joints, involving decalcification by incubating knee joints in 4% paraformaldehyde solution followed by phosphate buffer and then in 10% EDTA solution at 4°C for 2 weeks. Joints were embedded in paraffin wax and frontal sections (6 µm) cut at 200 µm intervals, followed by staining with haematoxylin, safranin-O/fast green. Ten sections from each mouse were graded using a validated scoring system,⁷ from 0 (normal) to 6 (>80% loss of cartilage) by two scorers blinded to the specimens, taking each quadrant of the joint separately and then

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summing scores. There was close agreement between scorers (intraclass correlation coefficient 0.97; 95% CI 0.95 to 0.98) with the mean difference in score being -0.05 (95% CI 0.01 to -0.11).

Assessment of osteosclerosis

From the sections obtained above, two were chosen at random, digitally captured (Olympus BX51, DP70, AnalySIS software) and stored for computerised planimetry analysis using QS Onscreen v 3.1.19, (E-quantities Ltd; <http://www.e-quantities.com>). Only two photomicrographs per mouse were required for planimetric analysis as osteosclerosis was relatively consistent across sections (coefficient of variation 2.1%). The area of the medial and lateral aspects of the tibial plateau was traced, from the epiphyseal plate to the lower zone of cartilage adjacent to subchondral bone. The tibial plateau region was selected as cartilage damage was maximal at the medial aspect with the lateral region never appearing to be affected in MMTL-sectioned wild-type mice, and so could be used as an internal reference. Controls were also performed in sham-operated as well as naive mice. Once the overall area on each side of the tibial plateau was determined, areas containing marrow were traced, summed and subtracted from the overall area on each side to yield the area occupied by bone. Comparisons were made by expressing as the percentage area occupied by bone in the medial tibial compared with the lateral region in order to normalise for animal size, because analysis of naive mice showed the difference between these areas to be minimal ($1.1 \pm 3.4\%$, mean \pm SEM, $n=5$). Scores were independently verified by another blinded assessor and there was good agreement between scorers (intraclass correlation coefficient 0.91; 95% CI 0.77 to 0.97) with the mean difference in score being 2.7% (95% CI 5.45 to -0.06). Osteosclerosis was not assessed in the ACL model as it was previously reported that bone erosion can penetrate to the epiphyseal plate.⁷

Immunohistochemistry

Following decalcification, sections were deparaffinised, rehydrated and stained with the murine monoclonal antibody SAM-11 (Santa Cruz Biotechnology, Santa Cruz, California, USA) at $1 \mu\text{g/ml}$ using the Dako (Ely, UK) animal research kit peroxidase. SAM-11 is a highly specific monoclonal antibody to human PAR-2 that binds the membrane-bound part of the receptor. Antigen-antibody complex was visualised utilising diaminobenzidine and counterstained with haematoxylin. The animal research kit was utilised to minimise the reactivity of secondary anti-mouse antibody with endogenous immunoglobulin.

Statistical analysis

Data were tested for normality (Sigmastat 2.03; SPSS Inc) and expressed as mean \pm SEM with comparisons by one or two-way ANOVA and multiple comparisons using Bonferroni correction. Non-normal data were transformed (square root) before statistical analysis and if normality was still not achieved, non-parametric methods were used. Agreement between scorers was assessed graphically using the Bland-Altman plot⁸ and expressed as the intraclass correlation coefficient.

RESULTS

Four weeks after MMTL section, immunohistochemical analysis of cartilage revealed increased PAR-2 expression in superficial layer chondrocytes (figure 1A), compared with sham-operated mice (figure 1B). Having established enhanced expression of the PAR-2 pathway with plausible tissue distribution, pathology

was compared in wild-type mice (PAR-2^{+/+}; $n=5$) and PAR-2-deficient littermates (PAR-2^{-/-}; $n=5$), with sham-operated wild-type mice ($n=4$) serving as a negative control. Compared with sham-operated mice (figure 1D), PAR-2^{+/+} mice showed cartilage damage and increased subchondral bone formation (figure 1E), whereas such changes were minimal in PAR-2^{-/-} mice (figure 1F). Such differences could not be attributed to an underlying difference in bone phenotype of PAR-2^{-/-} mice as the appearance of the unoperated knee did not differ from wild-type littermates (supplementary figure 1, available online only). To address the therapeutic utility of PAR-2, operated mice ($n=6$) were treated with SAM-11 antibody, which we previously demonstrated inhibits PAR-2 activation.⁹ In a further group ($n=5$), we utilised p520, a selective PAR-2 antagonist.¹⁰ Consistent with data in the PAR-2-deficient animals, mice treated with SAM-11 (figure 1G) or p520 showed significantly reduced osteoarthritis pathology 4 weeks postoperatively (figure 1H,I).

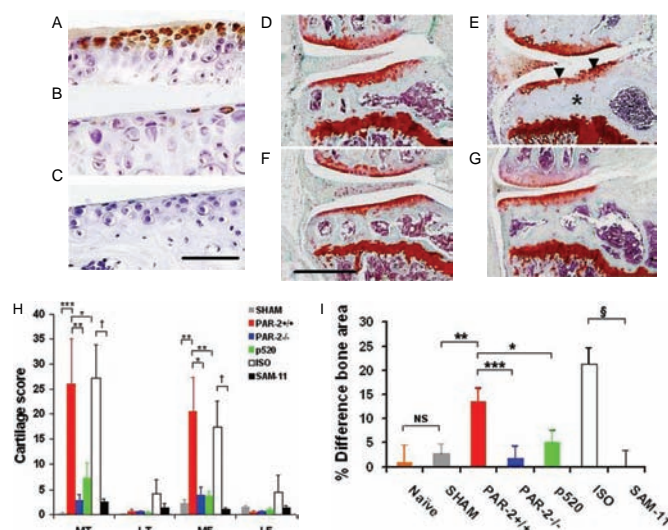


Figure 1 Protease-activated receptor 2 (PAR-2) absence or inhibition ameliorates osteoarthritis pathology in the 4-week medial meniscotibial ligament (MMTL) model. Immunohistochemistry of cartilage in the medial tibial aspect using SAM-11 to detect PAR-2 shows increased surface expression in chondrocytes of the wild-type mouse following MMTL section (A), compared with a sham-operated animal (B), with isotype control (C). Bar indicates $50 \mu\text{m}$. Histological appearances of the medial aspect of the knee joint 4 weeks after sham-operation (D), or following MMTL section in either a wild-type (E) or PAR-2-deficient mouse (F), or wild-type mouse treated daily with SAM-11 ($1 \mu\text{g}$ intraperitoneally) immediately before and after the induction of osteoarthritis (G). Arrowheads in (E) denote cartilage damage and asterisk indicates osteosclerosis. Scale bar in panel F indicates $500 \mu\text{m}$ and applies to all four photomicrographs. Cartilage degradation scores (H) for the medial tibial (MT), medial femoral (MF), lateral tibial (LT) and lateral femoral (LF) regions following MMTL section in PAR-2^{+/+} and PAR-2^{-/-} mice, PAR-2^{+/+} mice treated with PAR-2 antagonist p520 (5 mg intraperitoneally daily), SAM-11 ($1 \mu\text{g}$ intraperitoneally daily) or isotype (IgG2a) control antibody (ISO; $1 \mu\text{g}$ intraperitoneally daily), along with sham-operated controls. Data are expressed as mean \pm SEM, $n=4-6$. Analysis by one-way analysis of variance (ANOVA) with multiple comparisons using the Bonferroni correction, * $p<0.05$; ** $p<0.01$; *** $p<0.001$. $\dagger p<0.005$, Mann-Whitney rank sum test. Percentage difference in bone area (I) comparing the medial and lateral tibial regions following MMTL transection in the above groups of mice along with a naive control group. Analysis by one-way ANOVA with multiple comparisons using the Bonferroni correction, * $p<0.05$; ** $p<0.02$; *** $p<0.01$. $\S p<0.001$, t test. NS, not significant.

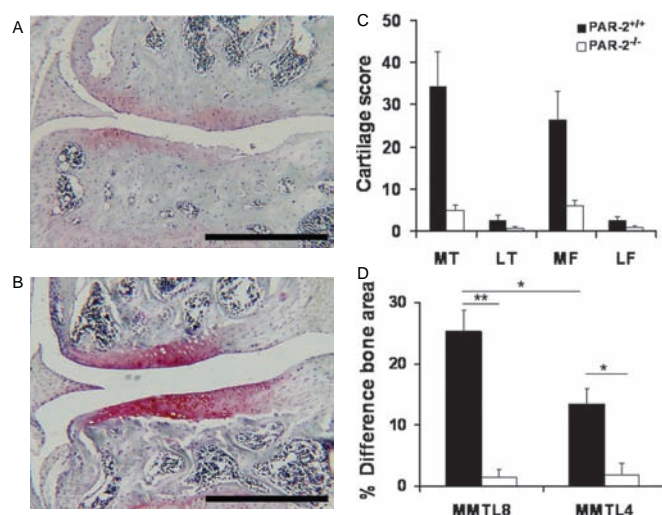


Figure 2 Protease-activated receptor 2 (PAR-2) deficiency still confers protection 8 weeks after medial meniscotibial ligament (MMTL) section. Examination of the medial tibial and femoral regions of the PAR-2^{+/+} mouse (A) compared with its PAR-2^{-/-} littermate (B) 8 weeks after MMTL section shows continued protection in the latter. Scale bars indicate 500 μ m. This was confirmed for these two groups (n=7 and 8, respectively) in terms of cartilage damage scores (C). **p<0.01, ***p<0.001. Osteosclerosis, comparing the percentage difference in bone area between the medial and lateral tibial areas, showed a significant increase in the 8-week model (MMTL8) compared with 4 weeks (MMTL4) in PAR-2^{+/+} mice but no difference between the PAR-2^{-/-} groups (D). *p<0.02, **p<0.0001. LF, lateral femoral; LT, lateral tibial; MF, medial femoral; MT, medial tibial.

Two-way ANOVA revealed a significant difference in cartilage degradation between groups (p<0.0001) and between regions (p<0.0001). PAR-2^{+/+} mice exhibited significantly greater cartilage degradation than sham-operated, PAR-2^{-/-} mice, or wild-type mice treated with p520 (figure 1H). Similarly, operated mice receiving an isotype antibody developed substantially greater cartilage degradation than SAM-11-treated mice. Consistent with previous work,⁷ cartilage damage only occurred at the medial aspects of the tibia and femur. Analysis of subchondral bone revealed differences between treatment groups (p=0.006, one-way ANOVA). PAR-2^{+/+} mice differentiated from sham-operated, PAR-2^{-/-} mice, and wild-type recipients of p520 (figure 1I). Consistent with this, mice receiving isotype control showed substantially greater osteosclerosis than SAM-11 recipients. Cartilage damage and osteosclerosis scores were significantly correlated (r=0.7, p<0.0001). As genetic deficiency of PAR-2, or inhibition by two distinct routes, namely targeting the protease cleavage site with SAM-11 or directly by receptor antagonism, proved effective in preventing the development of experimental osteoarthritis, we conclude that PAR-2 is a pivotal checkpoint in early progression of osteoarthritis pathology.

The longer-term effects of PAR-2 biology were examined using PAR-2-deficient mice by extending the postoperative period to 8 weeks. Compared with wild-type littermates (figure 2A) cartilage damage was still significantly (p<0.0001, two-way ANOVA, n=7–8) lower in PAR-2^{-/-} mice (figure 2B,C) and once again the latter showed no evidence of increased subchondral bone formation, whereas PAR-2^{+/+} mice showed a significant increase compared with the 4-week model (figure 2D). To test our hypothesis further, the more aggressive ACL transection

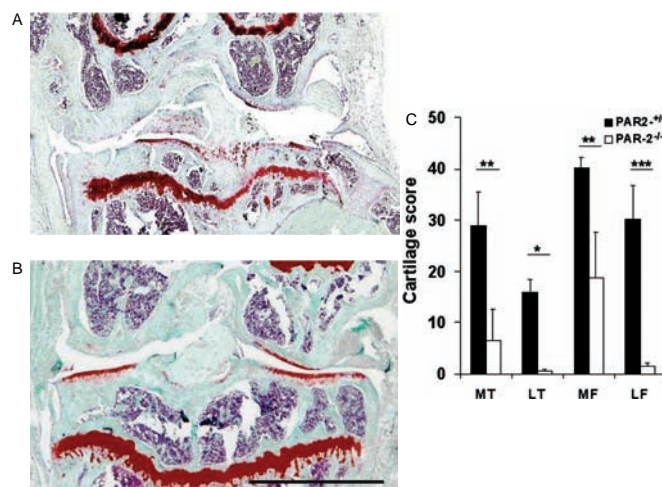


Figure 3 Eight weeks after anterior cruciate ligament transection, substantial damage was evident in multiple areas of the knee in protease-activated receptor 2 positive (PAR-2^{+/+}) mice (A), but less so in PAR-2-deficient (PAR-2^{-/-}) mice (B). Scale bar indicates 1000 μ m. Cartilage damage scores confirm the more extensive nature of damage in this model of arthritis, but there remain significant differences compared with PAR-2^{-/-} mice (C). ***p<0.001, **p<0.005, *p<0.05. LF, lateral femoral; LT, lateral tibial; MF, medial femoral; MT, medial tibial.

model was investigated. PAR-2^{+/+} mice developed almost complete cartilage erosion, particularly at the medial femoral condyle (figure 3A,C), whereas PAR-2^{-/-} mice exhibited significantly (p<0.0001, two-way ANOVA, n=7–8) lower levels of damage (figure 3B,C).

DISCUSSION

Our observations in PAR-2-deficient mice using both MMTL and ACL transection models provide powerful proof of concept for a key role of PAR-2 in joint degradation associated with osteoarthritis, and furthermore demonstrate the therapeutic potential of PAR-2 inhibition, which could have preventive clinical utility. The mechanism by which PAR-2 promotes osteoarthritis has yet to be clarified. PAR-2 provides for sensing by cells of the protease environment and allows them to respond subsequently. For example, PAR-2 regulates the synovial release of proinflammatory cytokines including interleukin-1 β ,¹¹ which is in turn a potent chondrocyte catabolin.¹² Activation of PAR-2 leads to the inhibition of osteoclast differentiation¹³ and could alter the balance between bone formation and resorption, and thereby contribute to osteosclerosis observed in PAR-2^{+/+} but not PAR-2^{-/-} mice. However, whether PAR-2 has a direct or indirect effect on the bone phenotype and its hierarchical role in osteoarthritis are areas for further investigation. Earlier studies revealed that selective PAR-2 activation of chondrocytes derived from human osteoarthritis cartilage leads to the generation of MMP-1 and MMP-13,⁵ both of which are known to play a key role in the degradation of aggrecan and collagens. Although the initiating event in the development of osteoarthritis lesions remains unknown, our data suggest that PAR-2 is a crucial checkpoint that mediates the effect of biomechanical instability upon cartilage degradation and osteosclerosis. PAR-2 inhibitors could therefore provide a new direction in the development of disease-modifying osteoarthritis drugs, which following disappointing results of trials with MMP inhibitors, bisphosphonates and cytokine inhibition,¹⁴ still remain elusive.

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