

# Proteinase-activated Receptor-2 Gene Disruption Limits the Effect of Osteoarthritis on Cartilage in Mice: A Novel Target in Joint Degradation

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**ABSTRACT.** *Objective.* Evidence indicates that proteinase-activated receptor (PAR)-2 participates in the degradative processes of human osteoarthritis (OA). We evaluated the *in vivo* effect of PAR-2 on articular lesions in a PAR-2-knockout (KO) mouse model of OA.

**Methods.** OA was surgically induced by destabilization of the medial meniscus of the right knee in C57Bl/6 wild-type (WT) and PAR-2 KO mice. Knee swelling was measured throughout the duration of the study (8 weeks postsurgery) and histologic evaluation of cartilage was done to assess structure, cellularity, matrix staining, and remodeling in the deep zone. Morphometric analysis of subchondral bone was also performed.

**Results.** Data showed significant knee swelling in the operated WT mice immediately following surgery, which increased with time (8 weeks post-surgery). Knee swelling was significantly lower ( $p \leq 0.0001$ ) in PAR-2 KO mice than in WT mice at both 4 and 8 weeks postsurgery. Cartilage damage was found in both operated WT and PAR-2 KO mice; however, lesions were significantly less severe (global score;  $p \leq 0.05$ ) in the PAR-2 KO mice at 4 weeks postsurgery. Operated WT mice showed reduced subchondral bone surface and trabecular thickness with significance reached at 4 weeks ( $p \leq 0.03$  and  $p \leq 0.05$ , respectively), while PAR-2 KO mice demonstrated a gradual increase in subchondral bone surface with significance reached at 8 weeks ( $p \leq 0.007$ ).

**Conclusion.** We demonstrated the *in vivo* implication of PAR-2 in the development of experimental OA, thus confirming its involvement in OA joint structural changes and reinforcing the therapeutic potential of a PAR-2 antagonist for treatment of OA. (First Release Feb 1 2011; J Rheumatol 2011;38:911–20; doi:10.3899/jrheum.100710)

*Key Indexing Terms:*

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Osteoarthritis (OA) is a slowly progressive disease resulting in erosion and loss of articular cartilage. Although the changes in cartilage are linked to a combination of mechanical and biochemical factors, significant evidence points to an association with inflammation, which is among the most significant changes that take place during the development of OA<sup>1</sup>. Studies have also revealed that, in the OA process, biological and morphological disturbances occur in the subchondral bone, and these alterations are suggested to be responsible for early pathological changes in cartilage<sup>2</sup>.

There is no known cure for OA, only symptomatic treatments. The most widely used therapeutics are analgesics, nonsteroidal antiinflammatory drugs, and anticycloxyge-nase (COX)-2 drugs. However, to date these drugs have not proven to reduce the joint structural modifications that occur during the OA process. Therefore, there is a need to identify new potential therapeutic targets able to arrest the disease progression, which would spare articular tissues and prevent symptoms including pain, swelling, and inflammation.

A member of a membrane receptor family, the proteinase-activated receptor (PAR), was reported to be

involved in the inflammatory process. These receptors belong to 7-transmembrane G protein-coupled receptors and are activated through a unique process of proteolytic cleavage. This cleavage requires a serine-dependent protease that unmasks an N-terminal sequence called a tethered ligand, which in turn binds to the receptor's second extracellular loop, thus activating the receptor's signaling cascade<sup>3</sup>. This receptor's unique activation method is an irreversible phenomenon<sup>4</sup>. This family is composed of 4 members (PAR-1 to PAR-4) expressed by several cell types and implicated in numerous physiological and pathological processes<sup>5</sup>. Although some members of the PAR family have recently been shown to be involved in inflammatory pathways, PAR-1 has been found to also be involved in arthritic tissues. Marty, *et al*<sup>6</sup> reported that the inhibition of thrombin, which is the main activator of PAR-1, reduced the development of arthritis in mice. In the same line of thought, Yang, *et al*<sup>7</sup> demonstrated that PAR-1<sup>-/-</sup> mice showed a significantly reduced severity of arthritis in an induced model. In joint inflammation, studies also provide strong evidence supporting an essential role of PAR-2 activation. Indeed, injection of a synthetic PAR-2 agonist was found to prolong joint swelling and synovial hyperemia in mice<sup>8</sup>. In contrast, intraarticular injection of PAR-2 small interfering RNA reduced arthritis in mice<sup>9</sup>. Moreover, in PAR-2<sup>-/-</sup> mice, the joint swelling in response to exogenous treatment with trypsin and tryptase, known activators of PAR-2, was absent<sup>9</sup>. Further, disruption of the proteolytic activation of PAR-2 by using a PAR-2 monoclonal antibody or a PAR-2 antagonist attenuated the joint inflammation<sup>9</sup>. In patients with rheumatoid arthritis, PAR-2, located in the synovial membrane, was predominantly found in endothelial cells of the synovium, in macrophage of the lining layer and sublining area, in mast cells of the sublining area, and in synovial fibroblasts<sup>10,11</sup>. Together, these data suggest a role of PAR-2 in the development of inflammatory joint diseases.

In OA pathology, PAR-2 has recently been documented in human cartilage/chondrocytes and synovial membrane/synovial fibroblasts<sup>12,13,14,15</sup>, with its expression levels significantly increased in OA compared to normal cells. In OA cartilage, PAR-2 activation has been associated with increased production of the matrix metalloproteinases (MMP)-1 and MMP-13, and COX-2<sup>13</sup>, well known key mediators of OA pathophysiology. Moreover, PAR-2 was also found to be present in human subchondral bone osteoblasts and was upregulated in OA<sup>16</sup>. In these OA cells, PAR-2-specific activation resulted in an upregulation of the level of membranous receptor activator of nuclear factor- $\kappa$ B ligand (RANKL) with no effect on osteoprotegerin production, as well as in an increase in bone resorptive activity<sup>16</sup>.

Our aim was to further investigate the *in vivo* effect of PAR-2 on the development of articular lesions. We explored the role of this receptor in PAR-2 knockout (KO) mice using a surgically induced model of OA. Histologic and morpho-

logical changes in the cartilage and in the subchondral bone were evaluated.

## MATERIALS AND METHODS

**Experimental animals.** Wild-type (WT) C57Bl/6 mice (Charles River Laboratories International Inc., Montreal, QC, Canada) and PAR-2 KO (PAR-2<sup>-/-</sup>) C57Bl/6 mice (The Jackson Laboratory, Bar Harbor, ME, USA) of both sexes were housed in wire cages in animal facilities with controlled temperature, humidity, and light cycles. They were allowed food and water *ad libitum*. The PAR-2 KO mice were generated from 3 homozygous PAR-2 KO reproducer pairs, which had a PAR-2 gene disrupted by the insertion of a construct containing hygromycin and neomycin selection genes. All the mice born from this breeding were genotyped for the disrupted PAR-2 gene. For each PAR-2 KO mouse generated, a piece of tail was subjected to a complete lysis with the DirectPCR Lysis Reagent and Proteinase K, following the manufacturer's instructions (Viagen Biotech Inc., Los Angeles, CA, USA). The polymerase chain reaction (PCR) was performed on the genomic DNA using the following primer sequences: for neomycin gene, 5'-CTT GGG TGG AGA GGC TAT TC-3' (antisense) and 5'-AGG TGA GAT GAC AGG AGA TC-3' (sense); and for PAR-2 gene, 5'-GGA TAG CCC TCT GCC TTT TC-3' (antisense) and 5'-GCA TTG AAC ATC ACC ACC TG-3' (sense). PCR amplification products were resolved by electrophoresis on a 2% agarose gel.

Mice used in this study were 8 weeks old, and they were distributed into 3 groups: control group consisting of nonoperated mice (WT, n = 11–17; PAR-2 KO, n = 8–22), operated mice sacrificed 4 weeks postsurgery (WT, n = 6–20; PAR-2 KO, n = 10–13), and operated mice sacrificed 8 weeks postsurgery (WT, n = 10–20; PAR-2 KO, n = 7–14). In a study using a sham mouse group of C57Bl/6 genetic background in which an opening of the knee capsule was performed at 8 weeks, no OA lesions of the articular tissues were observed, a finding that concurs with other studies<sup>17</sup>.

**Surgically induced OA mouse model.** OA was surgically induced in C57Bl/6 WT and PAR-2 KO mice by destabilization of the medial meniscus (DMM)<sup>18</sup>. The mice were anesthetized under isoflurane and O<sub>2</sub>, and the destabilization of the right knee joint was induced by transection of the anterior attachment of the medial meniscus to the tibial plateau. Immediately after surgery, an antibiotic ointment, silver sulfadiazine, was applied to the scar site and an analgesic, buprenorphine, was given postoperatively for 2 to 3 days. Mice were observed daily to verify healing and to ensure that they were using their right hind limb.

The day of OA induction was considered Day 0 (D0); the animals were sacrificed at 4 and 8 weeks postsurgery following a pentobarbital sodium injection. All mouse experiments were performed according to regulations established by the Canadian Council on Animal Care and were approved by the Animal Care Committee of the University of Montreal Hospital Centre.

**Knee joint swelling evaluation.** The mice of all groups (nonoperated and operated) were examined daily and knee diameter was measured in the mediolateral plane twice a week using a digital caliper (model 2071M, Mitutoyo Corp., Kawasaki, Japan)<sup>19</sup>. The swelling corresponded to the difference between joint diameter measured after surgery and joint diameter prior to surgery (D0).

**Histologic evaluation.** Mice were euthanized at 4 and 8 weeks after surgery or at equivalent age for the control (nonoperated) mice, and the entire knee joints were fixed in TissuFix #2 (Chaptec, Montreal, QC, Canada). The whole joint was decalcified in RDO Rapid Decalcifier for bone (Apex Engineering, Plainfield, IL, USA), and embedded in paraffin for histologic evaluation, as described<sup>20</sup>. Frontal sections of 5  $\mu$ m were stained with safranin-O, and 1 section per animal from the weight-bearing area of the joint was analyzed. Two independent observers blinded to group allocation graded the severity of the OA lesions in each specimen on a scale of 0 to 20 adapted from Mankin, *et al*<sup>21</sup>, and as described<sup>20,22</sup>. This scale was used to evaluate the severity of modifications based on surface/structural changes in cartilage (scale 0 to 5), cellular changes in cartilage (scale 0 to

4), the loss of cartilage matrix staining with safranin-O (scale 0 to 4), structure of the deep zone of cartilage (scale 0 to 4), and subchondral bone remodeling (scale 0 to 3). The final histologic score represented the most severe histologic changes within the cartilage lesions and subchondral bone on the medial tibial plateau.

**Subchondral bone histomorphometry.** Histomorphometry analysis was performed using a Leica DMLS microscope (Leica, Weitzlar, Germany) connected to a personal computer [Pentium IV-based, using OSTEO II Image Analysis Software (Bioquant, Nashville, TN, USA)]. The bone histomorphometry was performed on each specimen using a published method<sup>22,23</sup>. The section was chosen from the weight-bearing area of the joint, and, as in the other evaluations (histology and immunohistochemistry), 1 slide per animal was analyzed. From each specimen, 2 representative fields were identified ( $500 \times 250 \mu\text{m}$  boxes; original magnification  $60\times$ ). The lower limit of cartilage/subchondral bone junction was used as the upper limit of each field. The measurements and all histomorphometry data collected were as described<sup>22,23</sup>. The subchondral bone surface (percentage) and trabecular thickness ( $\mu\text{m}$ ) measurements were taken. The subchondral bone surface was determined by subtracting the bone surface lacuna area ( $\text{mm}^2$ ) from the total bone surface area ( $\text{mm}^2$ ). The measurements made for the 2 fields were then averaged for each specimen. Values for each section were considered separately for the purpose of statistical analysis.

**Immunohistochemistry.** Specimens were processed for immunohistochemical analysis, as described<sup>23,24</sup>. Serial sections were made from each specimen and 3 nonconsecutive sections from each specimen were processed for immunohistochemical analysis. Sections ( $5 \mu\text{m}$ ) of paraffin-embedded specimens were placed on Superfrost Plus slides (Fisher Scientific, Nepean, ON, Canada), deparaffinized in xylene, rehydrated in a reversed graded series of ethanol, and incubated in polyethylene glycol (0.3%) for 30 min. The specimens were then washed in phosphate-buffered saline (PBS) and in 0.3% hydrogen peroxide/PBS for 15 min. Slides were further incubated with a blocking serum (Vector, Burlingame, CA, USA) for 45 min, blotted, and then overlaid with the primary goat polyclonal antibody against PAR-2 (S-19; dilution 1:250; sc-8207, Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at  $4^\circ\text{C}$  in a humidified chamber.

Each slide was washed 3 times in PBS (pH 7.4) and stained using the avidin-biotin complex method (Vectastain ABC kit; Dako Diagnostic Inc., Mississauga, ON, Canada). This method entails incubation in the presence of the biotin-conjugated secondary antibody (anti-goat) for 45 min at room temperature followed by the addition of the avidin-biotin-peroxidase complex for 45 min at room temperature. All incubations were carried out in a humidified chamber and color was developed with a 3,3'-diaminobenzidine (Dako Diagnostic) containing nickel chloride. Slides were counterstained with eosin.

To determine the specificity of the staining, a control using the adsorbed immune serum (1 h at  $37^\circ\text{C}$ ) with a blocking peptide for PAR-2 (Santa Cruz Biotechnology) was performed according to the same experimental protocol. The control showed only background staining.

Each section was examined with a light microscope (Leica DMLS) and photographed with a QImaging Retiga camera (Surrey, BC, Canada).

**Statistical analysis.** Values are expressed as mean  $\pm$  SEM. Statistical analysis was performed using unpaired Student's *t* test or Mann-Whitney *U* test when appropriate. *P* values  $\leq 0.05$  were considered significant.

## RESULTS

**PAR-2 KO mouse genotyping.** The PAR-2 KO mice were genotyped to confirm that they had a disruption of the PAR-2 gene. The original PAR-2 fragment size is 200 bp, which should be 280 bp in the PAR-2 KO mice because of the hygromycin and neomycin insertion genes. The PAR-2 KO mice had a PAR-2 fragment of 280 bp as expected, while the WT mice showed a PAR-2 fragment of 200 bp

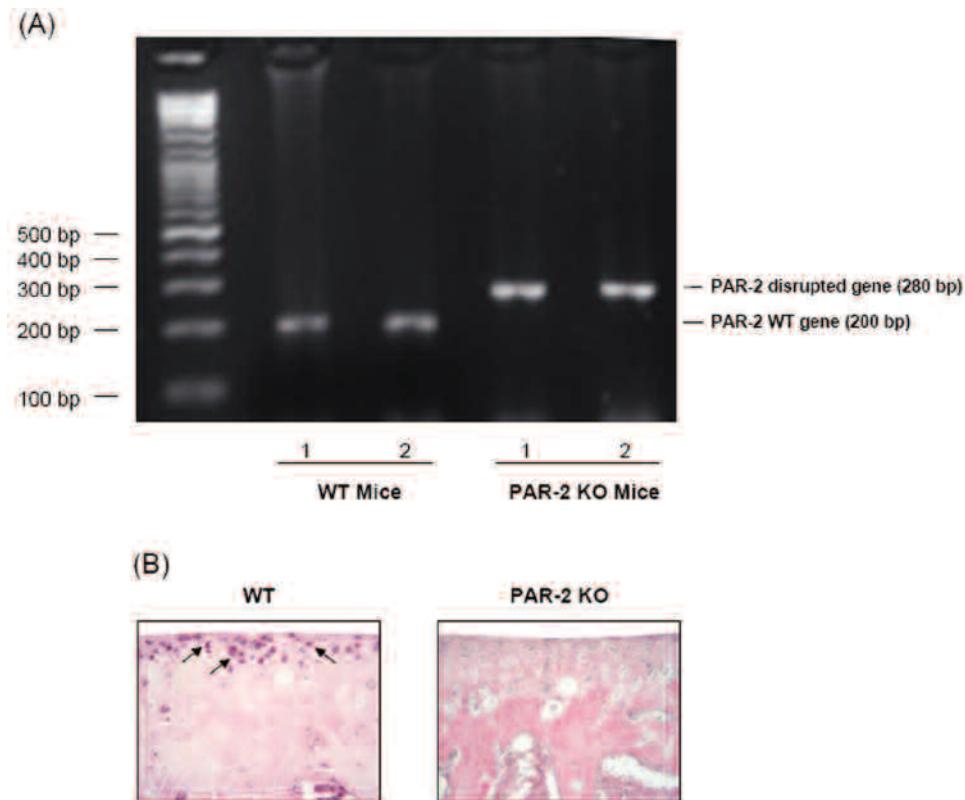
(Figure 1A). Moreover, the absence of PAR-2 was confirmed by the lack of PAR-2 immunostaining in the PAR-2 KO versus WT mouse tissue (Figure 1B).

**Knee joint swelling measurement.** To evaluate whether PAR-2 could be involved in the inflammatory process, joint swelling was measured in the right knee. Data showed that compared to the control WT mice (nonoperated), the operated WT mice developed significant joint swelling at D1 postsurgery (data not shown). The joint swelling was maintained until the end of the experiments, i.e., at 4 weeks (DMM 4W WT;  $p \leq 0.0001$ ) and 8 weeks (DMM 8W WT;  $p \leq 0.005$ ) postsurgery (Figure 2). Similarly, the operated PAR-2 KO mice, compared to the control PAR-2 KO (non-operated) mice, also developed joint swelling at D1 (data not shown) with a maximum reached at D7 ( $p \leq 0.0001$  for both KO groups; Figure 2). In contrast to the operated WT mice, starting at D7 a gradual reduction in joint swelling in the operated PAR-2 KO mice was noted, and values returned to the basal levels of the controls (nonoperated) at 4 and 8 weeks postsurgery (Figure 2). The right knee swelling of the WT mice remained significantly greater than that of the PAR-2 KO mice at all timepoints, and significance at 4 and 8 weeks postsurgery was  $p \leq 0.0001$  for both. Although the swelling observed immediately after surgery could be attributable to the surgery, the joint swelling in the operated PAR-2 KO mice remained significantly less than in the operated WT mice. Of note, as expected the operated sham group mice showed only a transient joint swelling (data not shown).

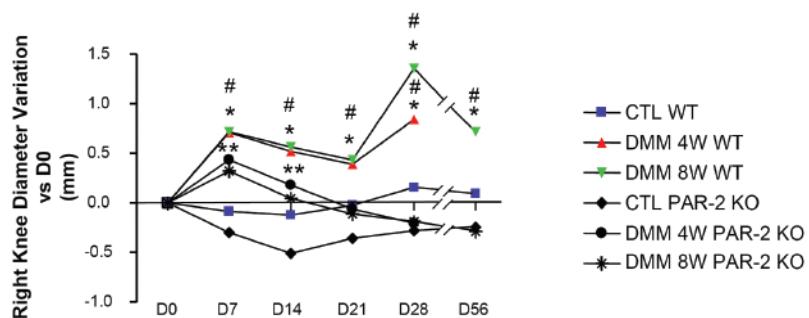
**Histologic analysis.** The effect of PAR-2 KO was further evaluated on cartilage and subchondral bone using histologic analysis. The global (total) histologic evaluation of the medial tibial plateau showed that both WT and PAR-2 KO mice developed significant OA lesions at 4 weeks ( $p \leq 0.002$  and  $p \leq 0.003$ , respectively) and 8 weeks ( $p \leq 0.04$  and  $p \leq 0.002$ ) postsurgery, compared to the nonoperated control group. However, these lesions were less severe in PAR-2 KO mice and a statistically significant difference was reached between WT and PAR-2 KO mice at 4 weeks ( $p \leq 0.05$ ) postsurgery (Figure 3A and 3B).

Data from the different criteria of the scoring scale showed that, compared to the nonoperated control mice, both WT and PAR-2 KO mice presented a significant alteration in cartilage surface at 4 weeks ( $p \leq 0.0004$  and  $p \leq 0.002$ , respectively) and 8 weeks ( $p \leq 0.02$  and  $p \leq 0.0006$ ) postsurgery (Figure 4A). However, there was significantly more damage in WT compared to PAR-2 KO mice at 4 weeks postsurgery ( $p \leq 0.003$ ; Figure 4A).

The cartilage cellularity of the operated WT mice, compared to the nonoperated control mice, demonstrated significant hypocellularity at 4 weeks ( $p \leq 0.003$ ), while cells from PAR-2 KO mice at 4 and 8 weeks postsurgery demonstrated less hypocellularity and were in clusters ( $p \leq 0.005$  and  $p \leq 0.02$ , respectively; Figure 4B). The cartilage cellu-



**Figure 1.** A. A representative analysis of semiquantitative RT-PCR of wild-type (WT) and disrupted PAR-2 fragment performed on two WT mice and two PAR-2 knockout mice. bp: base pair. B. Representative section of PAR-2 immunodetection in WT and PAR-2 KO unoperated mice (control; original magnification 400 $\times$ ). Arrows indicate PAR-2 staining on chondrocytes.

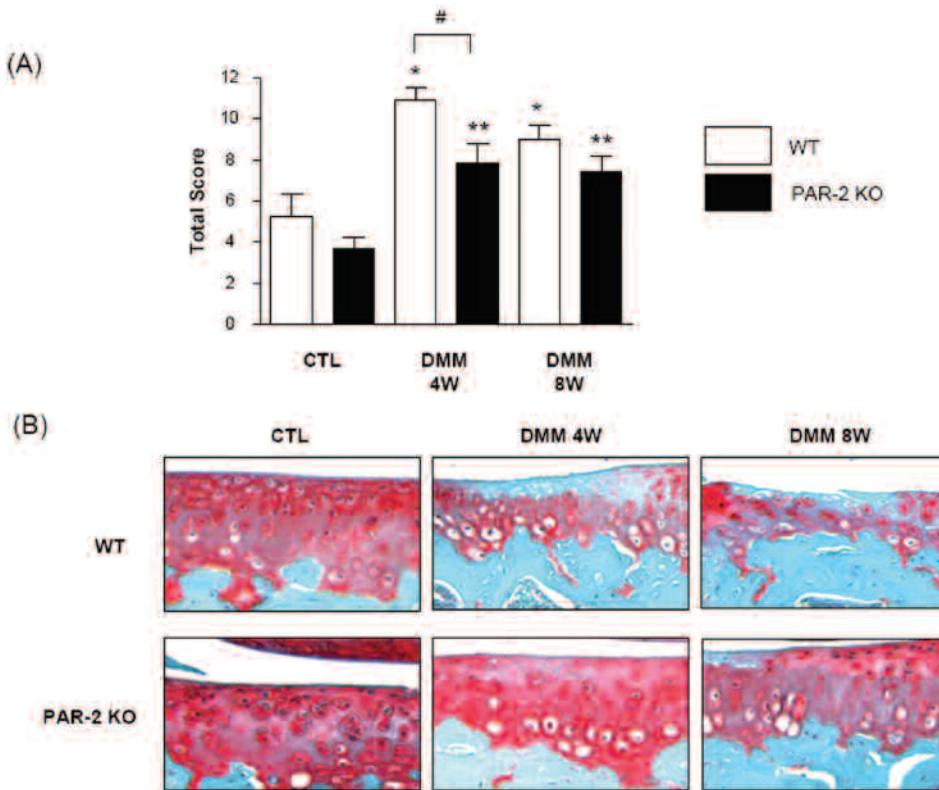


**Figure 2.** Right knee swelling in wild-type (WT) control (CTL; nonoperated) group ( $n = 17$ ), in WT operated (DMM) at 4 weeks (4W) group ( $n = 20$ ), in WT DMM 8W group ( $n = 20$ ), in PAR-2 knockout CTL group (nonoperated;  $n = 22$ ), in PAR-2 knockout DMM 4W group ( $n = 13$ ), and in PAR-2 KO DMM 8W group ( $n = 14$ ). Mice were examined daily and knee diameter (mm) measured using a digital caliper. The swelling corresponded to the difference between joint diameter measured at each timepoint and joint diameter prior to the experiment (D0). Statistical analysis was by unpaired Student's t test. P values  $\leq 0.05$  were the comparison between (\*) the WT CTL and WT DMM 4W or WT DMM 8W groups, between (\*\*) the PAR-2 KO CTL and PAR-2 KO DMM 4W or PAR-2 KO DMM 8W groups, and between (#) WT and PAR-2 KO groups.

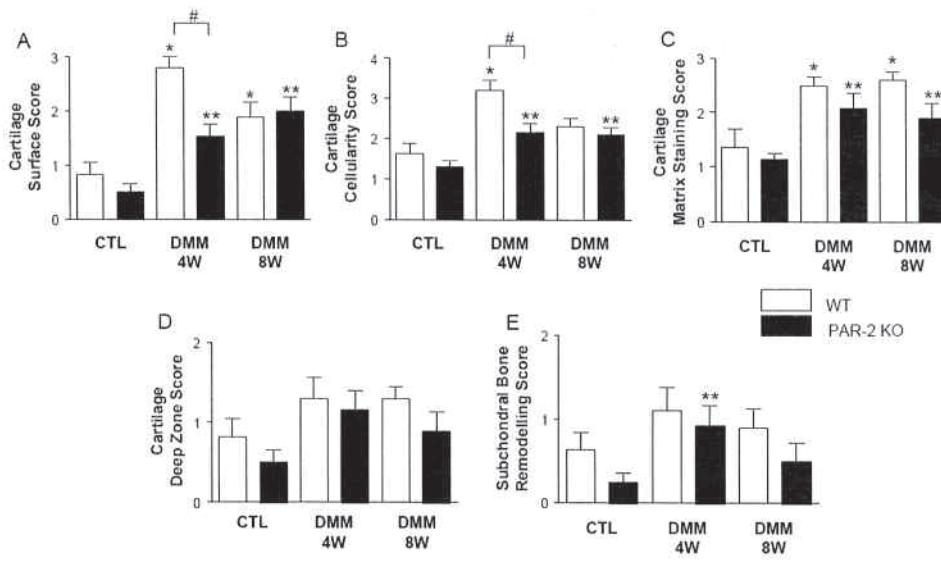
larity in the WT mice was significantly more affected than that in the PAR-2 KO mice at 4 weeks postsurgery ( $p \leq 0.02$ ; Figure 4B).

The cartilage matrix staining of the PAR-2 KO mice

scored lower than the WT mice, although both experimental groups showed significant loss of safranin-O staining at 4 weeks (WT,  $p \leq 0.03$ ; PAR-2 KO,  $p \leq 0.02$ ) and 8 weeks (WT,  $p \leq 0.02$ ; PAR-2 KO,  $p \leq 0.04$ ) postsurgery (Figure



**Figure 3.** Histologic analysis of the medial tibial plateaus 4 and 8 weeks postsurgery. A. Total score in wild-type (WT) control (CTL; nonoperated) group ( $n = 11$ ), in WT operated (DMM) at 4 weeks (4W) group ( $n = 10$ ), in WT DMM 8W group ( $n = 10$ ), in PAR-2 knockout (KO) CTL (nonoperated) group ( $n = 16$ ), in PAR-2 KO DMM 4W group ( $n = 13$ ), and in PAR-2 KO DMM 8W group ( $n = 10$ ). Data are mean  $\pm$  SEM and statistical analysis was by Mann-Whitney U test. P values  $\leq 0.05$  were the comparison between (\*) the WT CTL and WT DMM 4W or WT DMM 8W groups, between (\*\*) the PAR-2 KO CTL and PAR-2 KO DMM 4W or PAR-2 KO DMM 8W groups, and between (#) WT and PAR-2 KO groups. B. Representative sections of OA cartilage from the medial tibial plateaus of the study groups (safranin-O staining; original magnification 100 $\times$ ).



**Figure 4.** Detailed histologic analysis of cartilage and subchondral bone in wild-type (WT) control (CTL) group (nonoperated;  $n = 11$ ), in WT operated (DMM) at 4 weeks (4W) group ( $n = 10$ ), in WT DMM 8W group ( $n = 10$ ), in PAR-2 knockout (KO) CTL (nonoperated) group ( $n = 16$ ), in PAR-2 KO DMM 4W group ( $n = 13$ ), and in PAR-2 KO DMM 8W group ( $n = 10$ ). (A) cartilage surface score, (B) cartilage cellularity score, (C) cartilage matrix staining score, (D) cartilage deep zone score, and (E) subchondral bone remodeling score. Data are mean  $\pm$  SEM; statistical analysis was by Mann-Whitney U test. P values  $\leq 0.05$  were the comparison between (\*) the WT CTL and WT DMM 4W or WT DMM 8W groups, between (\*\*) the PAR-2 KO CTL and PAR-2 KO DMM 4W or PAR-2 KO DMM 8W groups, and between (#) WT and PAR-2 KO groups.

4C) compared to the nonoperated controls. However, significant difference was not reached between WT and PAR-2 KO mice.

In the deep zone of cartilage, the changes were greater in both experimental groups compared to their nonoperated control groups. Nevertheless, these did not reach statistical significance (Figure 4D).

At the subchondral bone level, the remodeling process in both groups was greater than that in the nonoperated control. However, PAR-2 KO mice at 4 weeks had significantly greater remodeling than the nonoperated PAR-2 KO control ( $p \leq 0.05$ ), but less than the operated WT mice at 4 weeks (Figure 4E).

*Subchondral bone histomorphometric analysis.* Finally, the subchondral bone surface and trabecular thickness were also evaluated (Figure 5). Data showed a significant reduction in bone surface in WT mice at 4 weeks postsurgery ( $p \leq 0.03$ ), while the PAR-2 KO mice had a significant increase in bone surface at 8 weeks postsurgery ( $p \leq 0.007$ ; Figure 5A). Of note, the nonoperated control PAR-2 KO mice had a significantly decreased percentage of bone surface compared to the nonoperated control WT mice ( $p \leq 0.0001$ ; Figure 5A).

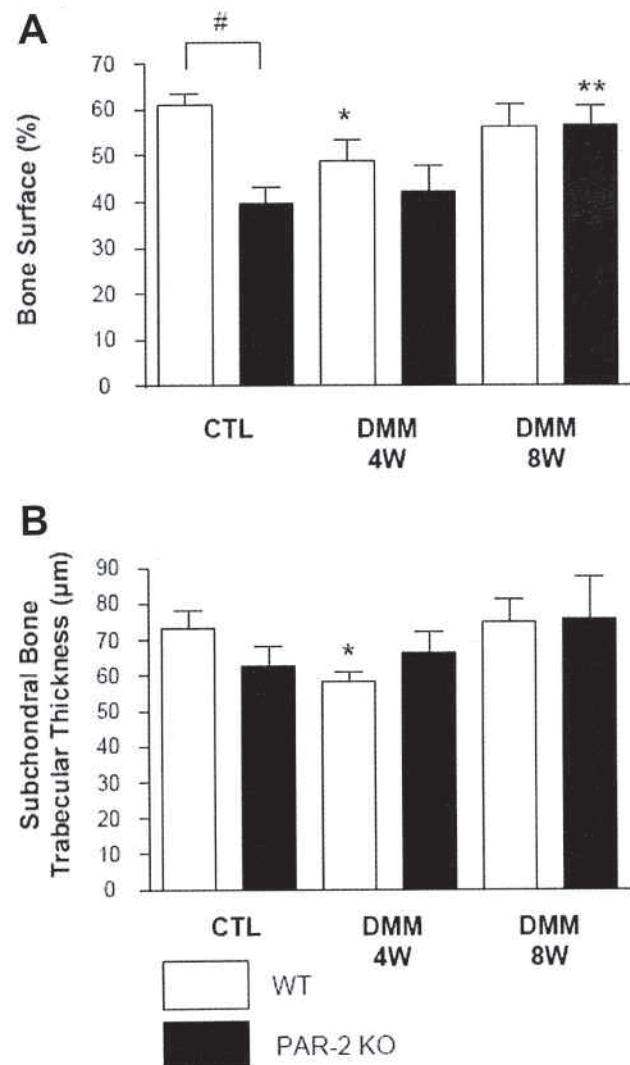
The pattern for the trabecular thickness was similar to that of the bone surface, in which the WT mice at 4 weeks post-surgery showed a significant decrease ( $p \leq 0.05$ ; Figure 5B), an effect that reverted at 8 weeks post-surgery. In contrast to the WT mice, the PAR-2 KO mice had increased trabecular thickness at 4 and 8 weeks post-surgery; however, this did not reach statistical significance.

## DISCUSSION

Although considerable advances have been made toward better understanding of the pathophysiological pathways in the OA process, much remains to be done to develop an effective disease-modifying OA drug that would reduce or stop the progression of the disease. In this context, new candidates that are able to target several joint tissues need to be identified.

Data from *in vitro* studies as well as our *in vivo* work strongly suggest that PAR-2 is an interesting candidate for an OA therapeutic target. Indeed, in OA tissues *in vitro*, PAR-2 activation was found to upregulate the synthesis of some inflammatory mediators and MMP in cartilage, and to increase RANKL production and resorptive activity in subchondral bone osteoblasts<sup>13,16</sup>, providing a critical link between inflammation and tissue remodeling/destruction. Our *in vivo* study using PAR-2 KO mice validates these *in vitro* findings and confirms the relationship between PAR-2 and the evolution of OA. PAR-2 thus appears to be a candidate in early OA.

The use of PAR-2 KO mice combined with a surgically induced OA model allowed us to study the chronological progression of this disease and the contribution of PAR-2 to the process. The OA model chosen, consisting of a destabi-



*Figure 5.* Histomorphometric analysis of subchondral bone in wild-type (WT) control (CTL; nonoperated) group ( $n = 11$ ), in WT operated (DMM) at 4 weeks (4W) group ( $n = 6$ ), in WT DMM 8W group ( $n = 10$ ), in PAR-2 knockout (KO) CTL (nonoperated) group ( $n = 8$ ), in PAR-2 KO DMM 4W group ( $n = 10$ ), and in PAR-2 KO DMM 8W group ( $n = 7$ ). The morphometric data presented are (A) the bone surface percentage of the subchondral bone, and (B) the trabecular thickness ( $\mu\text{m}$ ). Data are mean  $\pm$  SEM; statistical analysis was by unpaired Student's *t* test. P values  $\leq 0.05$  were the comparison between (\*) the WT CTL and WT DMM 4W or WT DMM 8W groups, between (\*\*) the PAR-2 KO CTL and PAR-2 KO DMM 4W or PAR-2 KO DMM 8W groups, and between (#) WT and PAR-2 KO groups.

lization of the knee by partial transection of the medial meniscus of the tibial plateau<sup>18</sup>, induces mild OA lesions, which mimic the first stages of the human disease.

The data showing that the PAR-2 KO mice had reduced joint swelling compared to the WT mice as early as D7, which was maintained over time, suggested that PAR-2 could be a mediator in the OA inflammatory process. Although the early measurements could have reflected the

wound healing, this was not the case later, as there were sustained significant differences between PAR-2 KO and WT. These data also agree with those of Kawabata, *et al*<sup>25</sup> and Ferrell, *et al*<sup>8</sup> using other models. The role of PAR-2 in OA inflammation could be related to its effect on COX-2 production, a factor known to be involved not only in the OA inflammatory pathways but also in the joint swelling process in induced arthritis models<sup>26,27</sup>. Moreover, the PAR-2 activation was shown to upregulate COX-2 in various human cell types<sup>28,29,30</sup> and in human OA cartilage<sup>13</sup>. Since the change observed in joint diameter could be a consequence of, or confounded by, other factors, e.g., bone remodeling, the implication of PAR-2 in inflammation requires additional studies for which another OA model should be used because, as reported by Glasson, *et al*<sup>31</sup>, the OA model used in this study did not show appreciable synovitis.

Because cartilage demonstrates a remodeling process in favor of catabolism during OA, we further explored the implication of PAR-2 in such a process. Data first showed that the operated PAR-2 KO mice had significantly less damage on the cartilage surface than the WT mice at 4 weeks postsurgery, suggesting that PAR-2 is involved in the structural changes in this tissue. These findings were recently supported by Ferrell, *et al*<sup>32</sup> who showed, using another OA-induced model (sectioning of the medial meniscotibial ligament), that the cartilage erosion seen in WT mice was substantially reduced in PAR-2 KO mice. A possible explanation for the PAR-2 effect on cartilage surface and cartilage matrix alterations could be its modulation of the production of some MMP. Indeed, in cartilage, among the important members of the MMP family, the collagenases MMP-1 and MMP-13 have been suggested to be highly involved in the development of OA<sup>33,34,35</sup>. Data further suggest that MMP-1 is involved mostly during the inflammatory process and MMP-13 during the remodeling process<sup>36,37,38</sup>. These 2 MMP could very well be involved, as recent data have revealed their induction in OA cartilage upon PAR-2 activation<sup>13</sup>.

In the cartilage from the operated WT mice, there was a high level of hypocellularity, while the PAR-2 KO mouse chondrocytes were still in clusters, indicating a delay in the development of the disease in the PAR-2 KO mice at 4 weeks postsurgery. Modulation and loss of cartilage cellularity is a feature of OA, and hypocellularity reflects cell death occurring by necrosis and/or apoptosis. Although the factors involved in cell death in cartilage are numerous, PAR-2 could be among them, as its activation was shown to lead to neuronal cell death in rats<sup>39</sup> through reactive oxygen species<sup>40</sup>, molecules known to be present in joint diseases<sup>41</sup>.

Imbalance in subchondral bone resorption/formation is a well described phenomenon in the pathogenesis of OA. Moreover, evidence suggests that subchondral bone alterations take place early during the OA process and that this

tissue plays a key role in cartilage degeneration in humans<sup>2,42,43,44</sup>, as well as in OA animal models<sup>23,45,46</sup>. In our *in vivo* study, the operated WT mice demonstrated subchondral bone remodeling at 4 weeks postsurgery, i.e., reduced subchondral bone surface and trabecular thickness, while at 8 weeks the subchondral bone surface and the trabecular thickness tended to revert to the basal level (control WT mice). Botter, *et al*, using a different strain, reported an increase in bone surface<sup>47</sup>. Although this appears to contrast with our result, one could speculate that at 4 weeks postsurgery, the WT mice demonstrated subchondral bone resorption that at 8 weeks progressed to subchondral bone sclerosis. To confirm this, this tissue would have to be evaluated after a longer period postsurgery.

The operated PAR-2 KO mice, compared to the nonoperated controls, exhibited no alteration in subchondral bone surface or trabecular thickness. This suggests that the PAR-2 KO induced less production of resorptive factors, which concurs with an *in vitro* study on human OA subchondral bone osteoblasts<sup>16</sup> in which PAR-2 activation increased the levels of RANKL and resorptive activity, as well as levels of MMP-1, MMP-9, and interleukin 6, factors actively involved in bone resorption<sup>44,48,49</sup>. Interestingly, at 8 weeks postsurgery, the PAR-2 KO mice showed a significant increase in subchondral bone surface and a trend toward increased trabecular thickness, to a level similar to that of the control WT mice (nonoperated). This observation supports the hypothesis of Song, *et al*<sup>50</sup> that the lack of PAR-2 favors the effect of another member of the PAR family, PAR-1, which may play a role in bone repair.

Data showed that the control PAR-2 KO mice exhibited a decreased subchondral bone surface compared to the control WT mice, suggesting a role for PAR-2 in the physiology of this tissue. This hypothesis is supported by a recent study<sup>51</sup> in which osteoblasts from PAR-2 KO mice compared to WT did not exhibit an increase in collagen type I messenger RNA expression, leading to a disorganization in bone matrix formation. This study also reported that these osteoblasts in the PAR-2 KO mice increased in number more rapidly than WT cells, an effect probably caused by decreased apoptosis rather than increased proliferation. These findings, along with our data, indicate that PAR-2 is an important receptor for bone matrix synthesis, and thus for bone formation.

The OA lesions, evaluated with the global histological score as well as some of the subcriteria of the scoring scale, although not statistically significant, showed a decreased score at 8 weeks compared to 4 weeks postsurgery. This could indicate recovery of the OA process at the latter time-point. Indeed, such a situation has been described for another induced-OA animal model, the monoiodoacetate intraarticular injection<sup>52</sup>, and the authors postulated that OA development is cyclic, with a reverse phase between 2 OA development phases. However, it cannot be excluded that the age

of the animals could have influenced the disease progression and that using younger mice could have favored a decrease in the score at 8 weeks postsurgery. Nonetheless, 8-week-old mice have already been used in this OA model<sup>17,53</sup>. We can then hypothesize that PAR-2 belongs to a gene group implicated in OA development, and that its disruption does not induce severe OA lesions as might be expected with this type of surgery in mice at 8 weeks postsurgery. Thus, in view of our overall results, we suggest that PAR-2 could be involved in early-stage OA (e.g., 4 weeks postsurgery) and possibly in the later stages of OA. However, to confirm this, a study of longer duration (e.g., 12 weeks postsurgery) is necessary. A limitation of our study is that the groups included both sexes, and in the DMM model, females develop less severe lesions than males<sup>54</sup>.

In OA, future therapeutic approaches should target not only reduction in cartilage degradation but also the subchondral bone remodeling process. Our *in vivo* study suggests that PAR-2 could act on these 2 different articular tissues. Hence, treatment with factors capable of neutralizing the membranous PAR-2 receptor could be of interest in limiting the production of cytokines and proteases involved in the catabolic process of the OA joint. Thus, it is noteworthy that some molecules that have demonstrated beneficial effects on responses in an inflammatory cell model<sup>55,56</sup> as well as in the prevention of development of OA lesions in dogs produced by instability by improving gait<sup>54</sup>, also reduce PAR-2 levels. Therefore, treatment targeting PAR-2 should contribute to an overall reduction in joint catabolism. Another potential therapeutic approach would be to target the factors responsible for PAR-2 activation. Among these factors, the serine proteases could be an interesting target. Indeed, according to the literature, this enzyme family is responsible for PAR-2 activation<sup>3,57</sup>. In OA cartilage, an important serine protease system is the plasminogen activator (PA) plasmin, of which the urokinase PA (uPA) plays a major role<sup>58,59</sup>. Interestingly, the uPA/PA system, in addition to acting directly on cartilage macromolecules, has been shown to be responsible for increased levels of other proteases, including collagenase<sup>58,60</sup>. The specific PAR-2 activation eliciting increased levels of MMP-1, MMP-13, and COX-2 in human OA cartilage<sup>13</sup> strongly suggests the likely involvement of this serine protease system in *in vivo* PAR-2 activation. Interaction between uPA and COX-2 was also shown in some cancer cells<sup>61,62</sup> as well as in corneal injury and inflammation<sup>63</sup>. HtrA1 could be another protease implicated in PAR-2 activation. This enzyme is known to be increased in OA tissues and to play a role in cartilage degradation<sup>64,65,66</sup>. Moreover, this protease cleaves its substrates at a specific site between arginine and serine, as trypsin does for the PAR-2 receptor<sup>3,57</sup>. Finally, a new protease involved in articular tissues, matriptase, could be a potential activator of PAR-2, since its importance has been shown in tissue matrix degradation in arthritic diseases<sup>67,68</sup>, as well as

its participation in PAR-2 activation in human cells<sup>69,70</sup>. Thus, identification of proteases that activate PAR-2 is of importance as their inhibition could be a useful therapeutic option.

Our study evaluated the *in vivo* histologic and morphological aspects of the effect of PAR-2 on OA cartilage and subchondral bone; our data confirm this factor's involvement in OA joint structural changes and reinforce the therapeutic potential of blocking PAR-2 for the treatment of this disease.

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