Cox-2 inhibition and the composition of inflammatory cell populations during early and mid-time tendon healing

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Summary

Background: During early tendon healing, the cells within the regenerating tissue are, to a large part, inflammatory leukocytes (CD45+). In a rat Achilles tendon healing model, the inflammation resolves between 5 and 10 days. In the same model, Cox inhibitors (NSAIDs) impair healing when given during the first 5 days, but have a positive effect if given later. We tested the hypothesis that a Cox inhibitor would exert these effects by influencing inflammation, and thereby the composition of the inflammatory cell subpopulations.

Methods: Achilles tendon transection was performed in 44 animals. Animals were randomized to either parecoxib or saline injections. Healing was evaluated by mechanical testing day 7 after surgery and by flow cytometry day 3 and 10.

Results: Cross-sectional area, peak force and stiffness were reduced by parecoxib 31, 33, and 25% respectively (p=0.005, p=0.002, and p=0.005). By flow cytometry, there was a strong effect of time (p<0.001) on virtually all inflammatory cell subpopulations (CD45, CD11b, CD68, CCR7, CD163, CD206, CD3, CD4), but no significant effect of parecoxib at any time point.

Conclusion: The results suggest that the negative effects of Cox inhibitors on tendon healing might be exerted mainly via mechanisms not directly related to inflammatory cells.

KEY WORDS: tendon healing, NSAID, inflammation, rat model, flow cytometry.

Background

Inflammation plays a pivotal role in the initiation of healing after tendon injury. Healing is also influenced by other factors, such as mechanical loading1. The regenerating tissue during early tendon healing is to a large part composed of leukocytes (CD45+). In a rat Achilles tendon transection model, the inflammation resolves between 5 and 10 days, as long as the tissue is at least partially protected from mechanical loading. Full loading prolongs the inflammatory phase of healing, increases strength and leads to dramatic changes in the composition of the leukocyte population2.

We have used a standardized rat Achilles tendon healing model in a series of previous studies related to inflammation2-4. In this model, mechanical loading or microtrauma by needling both regulate the expression of genes related to inflammation. The composition of leukocyte subpopulations, as studied by flow cytometry, was found to be remarkably similar between similarly treated animals, but highly variable over time and sensitive to degrees of mechanical loading2. This suggests that most factors that influence healing exert their effects primarily via an influence on inflammation.

In our tendon healing model, both mechanical strength and leukocyte compositions are strongly influenced by mechanical loading. Loading improves healing, while indomethacin and parecoxib (a selective Cox-2 inhibitor) both impair healing dramatically if given during the first 5 days, but have a slight positive effect if given later3, 5. Because of the strong link between improved healing and leukocyte composition in the tissue when loading was applied, we thought that other treatments with an effect on healing would also be linked to leukocyte composition. We therefore tested the hypothesis that a Cox inhibitor, parecoxib, would change the composition of the leukocyte population.

By analyzing different immune cell populations from healing tissue by flow cytometry, including different sub-populations of macrophages and T cells, we can describe how inflammation is influenced by NSAIDs. To our knowledge, no comprehensive study looking at effects of NSAIDs on the immune cell populations during tendon healing has been published. Although
we were mainly interested in changes of the entire pattern of immune cells, we needed to specify a specific, testable hypothesis. We therefore decided to choose a ratio between two key cell types, namely those expressing CCR7 (proinflammatory macrophages) and those expressing CD206 (anti-inflammatory macrophages) as the main outcome variable. This variable has previously been shown to be highly sensitive to mechanical loading<sup>2</sup>. The specific hypothesis of this work was that the ratio between pro- and anti-inflammatory macrophages, expressed as the ratio CCR7/CD206, would be influenced by parecoxib treatment.

Materials and methods

Study design

First, we confirmed the previous finding that parecoxib decreases the mechanical strength of the tendon. Female Sprague-Dawley rats (n=20; 11-12 weeks old) underwent Achilles tendon transection, and the tendon was allowed to heal spontaneously without suture. The rats were randomized to receiving parecoxib or saline. The day of surgery is regarded as day 0. Rats received a daily dose of parecoxib or saline day 0-4 and were euthanized for mechanical testing of the tendon on day 7 after surgery. There were 10 rats in each group. All animals were allowed free and fully loaded cage activity.

Thereafter, female Sprague-Dawley rats were used (n=24; 11-12 weeks old). The Achilles tendon was transected in all rats, as above. Rats were randomized to a daily dose of parecoxib or saline day 0-2 and were euthanized day 3 or received a daily dose of parecoxib or saline day 6-9 and were euthanized at 10. Immune cells from the healing tendons were evaluated by flow cytometry 3 and 10 days after surgery. There were 6 rats in each group. All animals were allowed free and loaded cage activity.

Drug administration

For mechanical evaluation, parecoxib was given 6.4 mg/kg body weight subcutaneously once a day (Dynastat, Pfizer, Belgium). Rats received parecoxib or saline injections day 0, 1, 2, 3 and 4. For the flow cytometric analysis, parecoxib was administered 6.4 mg/kg body weight subcutaneously once a day (Dynastat, Pfizer, Belgium). Rats for analysis on day 3, received parecoxib day 0, 1, and 2 and those for analysis on day 10, received parecoxib day 6, 7, 8 and 9. Control animals for the two groups received saline subcutaneously day 0, 1 and 2 or day 6, 7, 8 and 9. The injected parecoxib dose corresponds to the postoperative dose per body weight for humans with adjustment for a factor 4 difference metabolic rate.

Mechanical testing

Rats were euthanized by CO<sub>2</sub>. The Achilles tendon with calcaneal bone and gastrocnemius and soleus muscle was harvested. The muscle was scraped off and tendon fibers were fixed by sand paper in a metal clamp. The calcaneal bone was fixed in a custom-made clamp 30° at dorsiflexion relative to the direction of traction. The mechanical testing machine (100R, DDL, Eden Prairie, MN) pulled the mounted tendon at constant speed (0.1 mm/s) until failure. Peak force at failure and stiffness were calculated by the testing machine. Sagittal and transverse diameter of the mid part of the callus were measured by a caliper and cross-sectional area was calculated by assuming an elliptical geometry as described previously<sup>4</sup>. Measurements were performed by a blinded investigator.

Tissue harvest and retrieval of single cells

Rats were euthanized by CO<sub>2</sub> and the tendons retrieved. To ensure excision of only newly formed healing tissue between the resection ends, two cuts were made, 4 mm apart, perpendicular to the direction of the tendon in the middle of the former defect. The excised specimens were placed in digestion buffer (RPMI 1640 with, 5% heat inactivated fetal bovine serum, and 10 mM HEPES). The specimens were minced into small pieces and incubated with 1 mg/ml Collagenase D (Roche) and 30 μg/ml DNase (Roche) at 37°C for 45 min. Specimens were passed through a 70 μm cell strainer (Fisher scientific). Single cells were washed, and red blood cell was removed using ACK lysis buffer (155 mM NH<sub>4</sub>Cl, 10 mM KHCO<sub>3</sub>, and 88 μM EDTA). Trypan blue (Life technologies) was used to count live cells<sup>2, 7, 8</sup>. Flow cytometric phenotyping of immune cells

Antibodies were CD45 (leukocyte), CD3 (T cell), CD4 (T helper cell), CD25 and Foxp3 (regulatory T cell), CD8a (cytotoxic T cell) from Biolegend and CD11b (phagocyte), CD68 (pan-macrophage) from AbD serotec and CCR7 (M1 macrophage), CD206 (M2 macrophage) from Bioss and CD163 (M2 macro-
phage) from LSBio. Single cell suspensions were first stained for the surface markers CD45, CD11b, CD163, CD206, CCR7 (macrophage panel), or CD45, CD3, CD4, CD25, CD8 (T cell panel). Cells were then stained for intracellular staining (CD68, Foxp3) as previously described. Data were acquired using Gallios flow cytometer (Beckman Coulter) and fluorescence minus one (FMO) samples were used to set the gates. Evaluation was performed using FlowJo v10 software (Supplementary Figure).

**Statistics**

The mechanical parameters were tested with Student’s t-test, and peak force was regarded as the main mechanical outcome. The flow analysis, the specific hypothesis was that the ratio CCR7/CD206 would be influenced by parecoxib treatment. This was analyzed with two-way ANOVA using CCR7/CD206 ratio as dependent, and time and drug treatment as independent variables. Descriptive data (ratios for other markers) are shown with 95% confidence inter-
vals for the ratio between parecoxib and controls, without correction for multiplicity.

### Results

As expected, the mechanical strength day 7 was significantly reduced by parecoxib (Tab. I, Fig. 1). One rat was excluded before testing, due to a wound problem.

The M1/M2 ratio (CCR7/CD206) was not affected by the drug treatment in total or at any time point (Fig. 2). In contrast, the ratio diminished by a factor of 2 from day 3 to day 10 (p<0.001).

Looking at all cell subpopulations at the two time points separately, no significant effect of parecoxib could be seen, and the pattern of cell composition appeared quite similar between the parecoxib and control groups at each time point, but different between day 3 and day 10 (Fig. 3). The leukocytes (CD45+) constituted 60% of all cells day 3, but only 12% day 10 in saline and parecoxib treated animals.

At day 3, changes by more than a factor of 2 due to parecoxib can be excluded with high confidence for any cell subpopulation (Fig. 4). At day 10, the confidence intervals were wider, but still without signs of a treatment effect. In contrast, there was a strong effect of time on virtually all cells (CD45, CD11b, CD68, CCR7, CD163, CD206, CD3, CD4; p<0.001 for all) (Fig. 3). They all decreased over the time.

### Discussion

NSAIDs have a deleterious effect if given during the early inflammatory phase of tendon healing in the rat, and a positive effect if given during the later remodeling phase\(^3\). We hypothesized that this effect might be related to an influence on the immune cell populations during tendon healing. Surprisingly, we found only small and statistically non-significant effects on the immune cell populations in spite of the fact that we have repeatedly shown this treatment to strongly decrease the strength of the healing tissue\(^3\).\(^4\). This makes it unlikely that NSAID effects on tendon healing are exerted by dramatic increases or decreases of the number of cells within the studied leukocyte subpopulations.

In spite of the name “anti-inflammatory”, not much is known about NSAID effects on inflammatory cell responses during healing. In a model using collagenase to induce tendon injury, diclofenac reduced the accumulation of neutrophils and CD68 macrophages by
Figure 3 A, B. Inflammatory cells during tendon healing 3 and 10 days after tendon transection with parecoxib or saline. All markers are expressed as percent of all cells. There was a significant effect of time (p<0.001) on the following inflammatory cell subpopulations (CD45, CD11b, CD68, CCR7, CD163, CD206, CD3, CD4). They diminished from day 3 to day 10.

A) Bar graphs separated between macrophages (day 3 and day 10; to the left) and T cells (day 3 and day 10; to the right). For simplicity, CD45 is also shown in the macrophage bar graph. At day 3, cells labelled with CD25, and Foxp3 were too few in both groups for an accurate analysis.

B) Signature plot illustrating the same data as in (A). This plot illustrates the similarity in pattern between animals in each group: each curve connects different markers from the same rat. Days after transection: 3 and 10. P: parecoxib. S: saline. The points on the line represents the immune cell markers in the following order: CD45 (leukocytes), CD11b (phagocytes), CD68 (pan-macrophages), CCR7, CD163, CD206 (macrophage subtypes), CD3, CD4, CD25, Foxp3, CD8a (T cell and T cell subtypes). Vertical axis log scale. N = 6 for all groups.

Figure 4. Difference between parecoxib and saline in the number of marker positive cells out of all cells. 95% confidence intervals for the differences are shown. Values are expressed as a ratio parecoxib/saline. At day 3, cells labelled with CD25, and Foxp3 were too few in both groups to perform accurate analysis.
59 and 35% in the paratenon, but there was no effect on their numbers in the tendon core\textsuperscript{6}. In pressure ulcers, celecoxib diminished the neutrophils at 3 days and macrophages at 3 and 7 days after ulceration\textsuperscript{10}. In a full thickness skin wound healing model, celecoxib also reduced the number of neutrophils 48 hours after surgery\textsuperscript{11}. 

\textbf{Conflict of interest}

No conflicts of interest, financial or otherwise, are declared by the Author(s).

\textbf{References}


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