

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 later^{3, 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 loading².

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 loaded and had free cage activity. The rats were housed 2 or 3 per cage with 12:12 light and given food and water ad libitum. All experiments were approved by the Regional Ethics Committee and were in accordance with the MLTJ guidelines (15-15)⁶.

Surgery

Rats were anesthetized with isoflurane gas (Forene, Abbot Scandinavia, Solna, Sweden) and received antibiotics (25 mg/kg, Oxytetracycline, Engemycin; Intervet, Boxmeer, The Netherlands) preoperatively and analgesics (0.045 mg/kg, Buprenorphine, Temgesic; Schering-Plough, Brussels, Belgium) were given subcutaneously pre and postoperatively. The skin on the right Achilles tendon was shaved and cleaned with chlorhexidine ethanol. Thereafter, a transverse skin incision was made lateral to the Achilles tendon. The plantaris tendon was removed and the Achilles tendon was cut transversely and the tendon was left unsutured to heal spontaneously. Finally, the skin was sutured with two stitches.

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 CO₂. 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 previously⁴. Measurements were performed by a blinded investigator.

Tissue harvest and retrieval of single cells

Rats were euthanized by CO₂ 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 NH₄Cl, 10 mM KHCO₃, and 88 µM EDTA). Trypan blue (Life technologies) was used to count live cells^{2, 7, 8}.

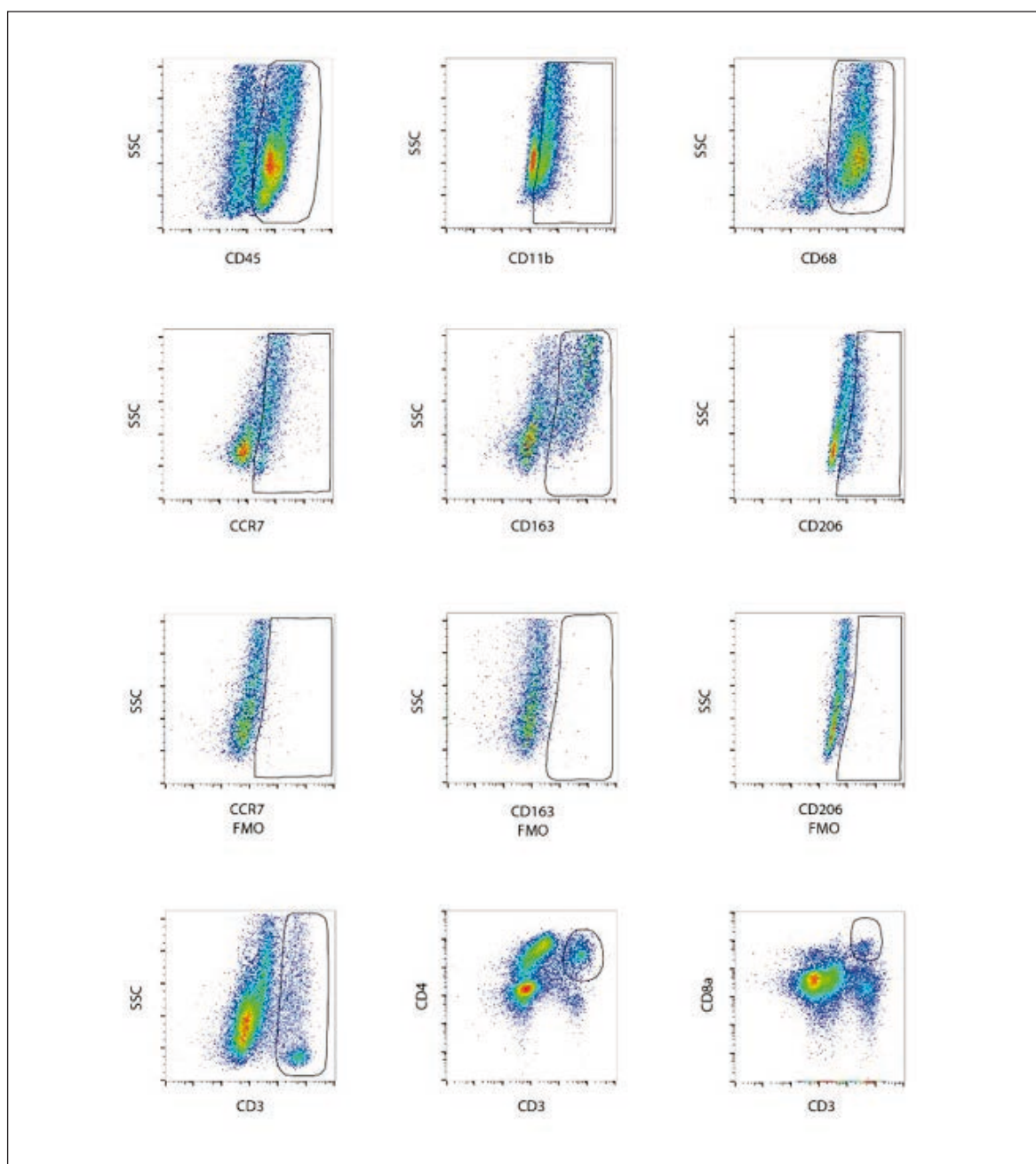
Flow cytometric phenotyping of immune cells

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 described². 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-



Supplementary Figure. Representative flow cytometry plots.

Table I. Mechanical properties of rat Achilles tendons 7 days after transection.

	Saline	Parecoxib	Diff %	p-value	95% CI
Peak force, N	16.3 ± 3.7	10.9 ± 2.9	33	0.002	13 to 53
Stiffness, N/mm	2.4 ± 0.5	1.8 ± 0.4	25	0.005	8 to 42
Cross sectional area, mm ²	10.6 ± 2.3	7.3 ± 2.2	31	0.005	10 to 52

Mean ± SD. Difference and 95% confidence interval for the difference between group means are given as percent of saline mean.

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

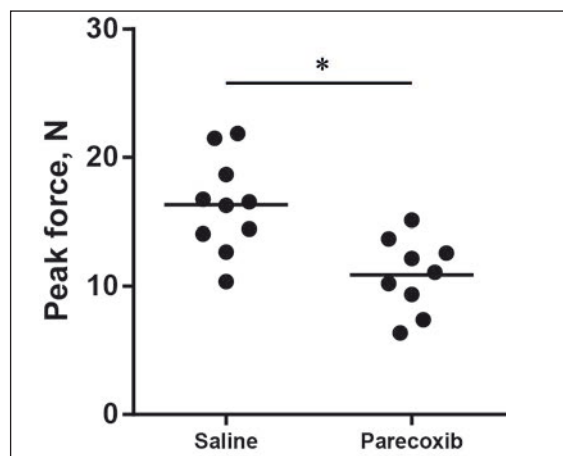


Figure 1. Peak force at failure 7 days after tendon transection. Peak force was reduced by parecoxib ($p = 0.002$) at day 7.

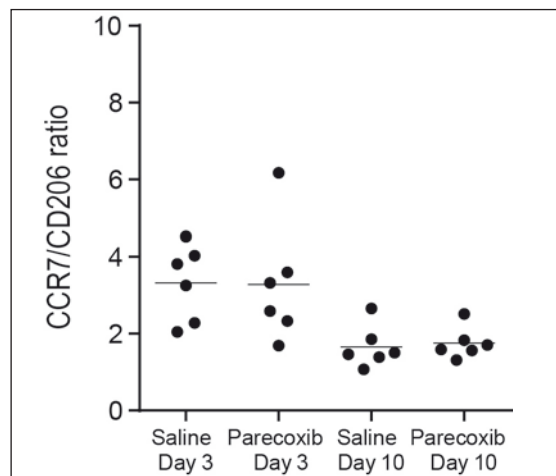


Figure 2. M1/M2 ratio (CCR7/CD206 among CD11b+ CD68+ cells) 3 and 10 days after surgery. The M1/M2 ratio was not significantly affected by parecoxib treatment at any time point.

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 phase³. 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

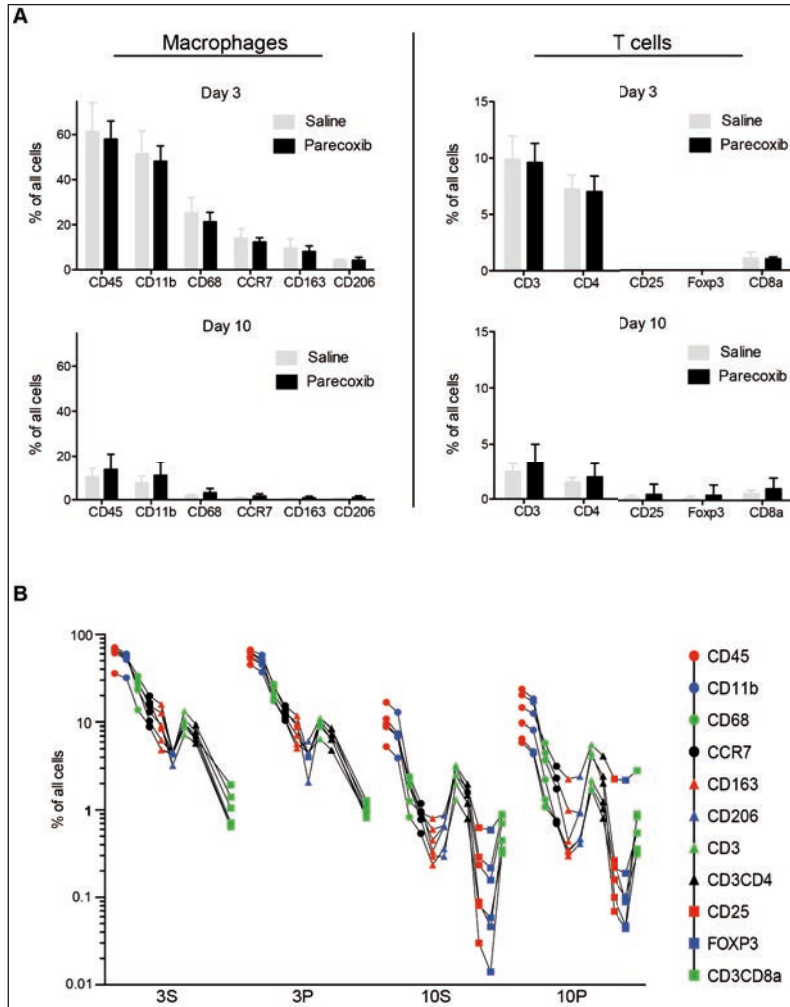


Figure 3 A, B. Inflammatory cells during tendon healing 3 and 10 days after tendon transection with parecoxib or saline. All markers are expressed as percent of all cells. There was a significant effect of time ($p < 0.001$) on the following inflammatory cell subpopulations (CD45, CD11b, CD68, CCR7, 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.

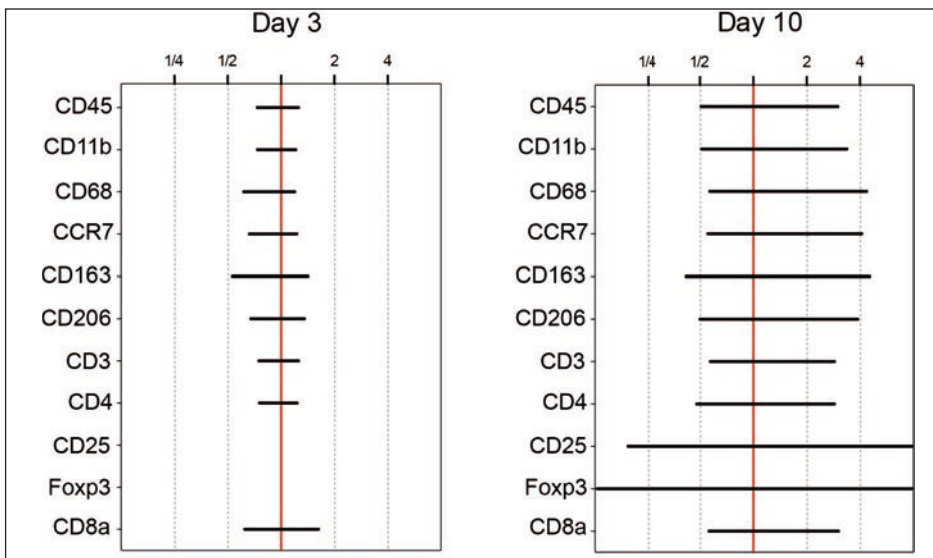


Figure 4. Difference between parecoxib and saline in the number of marker positive cells out of all cells. 95% confidence intervals for the differences are shown. Values are expressed as a ratio parecoxib/saline. At day 3, cells labelled with CD25, and Foxp3 were too few in both groups to perform accurate analysis.

59 and 35% in the paratenon, but there was no effect on their numbers in the tendon core⁹. In pressure ulcers, celecoxib diminished the neutrophils at 3 days and macrophages at 3 and 7 days after ulceration¹⁰. In a full thickness skin wound healing model, celecoxib also reduced the number of neutrophils 48 hours after surgery¹¹.

PGE₂ is one of the most studied end products of a synthesis pathway being blocked by NSAIDs. PGE₂ can regulate the differentiation and migration of T cells. Dendritic cells need to migrate to lymph nodes to present antigen to T cells. This migration is poor, unless dendritic cells are exposed to PGE₂ during their maturation^{12, 13}. This is one of the few described connections between NSAIDs and immune cells.

NSAIDs inhibit healing of injured bone or tendon in many different animal models, and the deleterious effect on fracture healing have even been shown in a randomized clinical trial¹⁴. NSAIDs inhibit Cox enzymes, thereby decreasing the synthesis of prostaglandins, most notably PGE₂. PGE₂ has four different G-protein coupled cell surface receptor subtypes (EP1, EP2, EP3, EP4), which mediate their pharmacological activities. Stimulation of these receptors by a non-prostanoid EP2 receptor-selective agonist can increase the proliferation and differentiation of mesenchymal cells into osteoblasts^{15, 16}. Another agonist, has similar effects on EP4 receptors, increasing the activity and number of osteoblasts¹⁷.

It seems possible that similar EP2 and EP4 dependent mechanisms might stimulate also tendon healing via direct effects on mesenchymal cells. This could explain why parcoxib influences strength without an obvious effect on inflammation. The deleterious effect of early NSAID treatment seems independent of effects on immune cell subpopulations.

The literature on the effects of NSAIDs on tendon healing seems inconsistent. Some reported reduced load to failure and tensile strength of the healing tendon^{4, 18} while other studies describe improved biomechanical properties^{5, 19}. These discrepancies are probably due to timing issues, as it has been shown that NSAIDs have a detrimental effect in the early inflammatory phase, but a slight positive effect during remodeling³. NSAID treatment had an inhibitory effect on migration and proliferation of tendon cells in culture but did not affect the collagen expression^{20, 21}.

One of the limitations of this study is that we have not studied unloaded tendons, as in the human situation with a braced ankle. Moreover, the difference in size between rats and humans could have a profound influence on healing mechanisms. As there is no data on the effects of NSAIDs on human tendon healing, the implications of this study for the human situation are unclear.

In conclusion, it is possible that NSAIDs, in spite of their name “anti-inflammatory”, exert their effects on tendon healing mainly via mechanisms not directly related to inflammatory cells.

Conflict of interest

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

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