Macrophage populations and the Lipoxin A<sub>4</sub> receptor in tendinopathy

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INTRODUCTION: There has been much debate about the importance of inflammation in the development of tendinopathy. Although transient low grade symptomatic inflammation and marked cellular infiltration occur in early injury, the characterisation and contribution of inflammatory components to the development of tendinopathies are not fully understood. The aim of this study was to determine if active inflammation occurs in naturally occurring flexor tendon injury. This was investigated by determining changes in macrophage (Mφ) sub-populations in normal and injured equine tendons at different stages of injury. The presence of the Lipoxin A<sub>4</sub> receptor (FPR2/ALX) was also assessed as a marker for resolving inflammation during tendon healing.

MATERIALS AND METHODS: Post mortem tissues were harvested from normal (n=5), sub-acute (3-6 weeks post injury; n=5) and chronic (>3 months post injury; n=5) equine superficial digital flexor tendons (SDFT) from horses between 4-16 years of age. Tendon sections were snap frozen and 8-10μm cryosections cut. Immunofluorescent staining was performed to characterise Mφ sub-populations in cryosections of normal and injured tendons on the basis of expression of the following surface antigens: CD172a (pan Mφ), pro-inflammatory CD14 (M1Mφ) and anti-inflammatory CD206 (M2Mφ) polarised phenotypes, in addition to FPR2/ALX. Expression levels for each marker were determined by calculating the ratio of areas of immunopositive cells to counterstained nuclei for both pan Mφ and FPR2/ALX antibodies. For the Mφ dual labeling method, a ratio of areas of positive immuno-reactivity for CD14 and CD206 was used to determine the predominant Mφ polarity in sub-acute and chronic tendon injuries. To assess the effects of pro-inflammatory mediators on FPR2/ALX expression, SDFT explants from 3 normal horses aged between 2-15 years were stimulated in vitro with either 5ng ml<sup>-1</sup> IL-1β, 1.0μM PGE<sub>2</sub> or a combination of both mediators. Non-stimulated samples served as controls. Explants were harvested immediately (time 0) and 12, 24 and 72 hours after stimulation, snap frozen and embedded. Cryosections were probed with FPR2/ALX antibody for each experimental condition and time point and quantitative analysis performed on immunopositive cells.

RESULTS: Normal tendons did not stain for either of the Mφ markers or FPR2/ALX. In contrast, Mφ were found in both sub-acute and chronically injured tendons, with significantly increased Mφ and FPR2/ALX expression in sub-acute injured SDFT (P=0.008 and 0.001, Fig.1A & 1B respectively). Furthermore, double staining revealed that pro-inflammatory M1Mφ predominated in sub-acute injuries (Fig.1B), whereas a phenotype switch to M2Mφ polarity was observed in chronic tendon injuries (P<0.001) (Fig.1C & D). In addition, FPR2/ALX expression was upregulated by pro-inflammatory mediators in tendon explants in vitro (Fig.2B). FPR2/ALX expression was at baseline levels between 0-72 hours in non-stimulated control explants. However, with stimulation with IL-1β or PGE<sub>2</sub> upregulated tenocyte FPR2/ALX expression, with maximal expression occurring at 72 hours (P<0.002 and 0.01 respectively). Combined stimulation with both mediators also increased FPR2/ALX expression at this time point (P=0.02); however no additive effect was observed.

DISCUSSION: The crucial role of Mφ as effectors of tissue injury and repair are well documented in other healing connective tissues (1, 2). In the present study, the greater proportion of M1Mφ in sub-acute compared to chronic injury reflects the pro-inflammatory status of recently injured tendon. Although not previously reported in tendon, FPR2/ALX expression was upregulated on tenocytes during sub-acute injury. This may suggest active resolution in an attempt to control the extent of inflammation and limit the degree of damage to tendon extracellular matrix. Indeed, both, IL-1β and PGE<sub>2</sub> significantly upregulated FPR2/ALX protein expression in tendon explant cultures, but interestingly showed no additive effect, suggesting that transcription or translation of FPR2/ALX is tightly regulated. The M2Mφ phenotype predominated in chronic injury; these cells were located in peri-vascular endotenon regions and at the interface between normal and healing tendon. We speculate that presence of M2Mφ in chronic tendon injury with concurrent reduced FPR2/ALX expression represent failure to adequately resolve inflammation, which may increase the propensity for fibrous tendon repair in contrast to Mφ deficient wounds that heal without scar formation (3, 4). Taken together, our data suggests that although tenocytes are capable of mounting a protective mechanism to counteract inflammatory stimuli, this is of insufficient duration and magnitude in natural tendon injury, which may potentiate chronic inflammation and fibrotic repair, as indicated by the presence of M2Mφ.

REFERENCES
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Box plot illustrating ratio of areas (μm<sup>2</sup>) of fluorescent: counterstained nuclei corresponding to (A) CD172a pan Mφ in normal (uninjured), sub-acute and chronic injured equine tendons; (B) CD14 (M1Mφ) and (C) CD206 (M2Mφ) expression in sub-acute and chronic injured tendons. (D) Log transformed ratio of immunopositive areas of M1M2Mφ from dual labeled CD14 and CD206 sections of sub-acute and chronically injured tendons. SAI= sub-acute injury (n=5), CI= chronic injury (n=5), N= normal tendon (n=5). All values represent median with maximum and minimum range. ***P<0.001, **P<0.01, *P<0.05.

Fig.1: Double immunostaining of SDFT reveals a shift in Mφ polarity. Box plots illustrating ratios of areas (μm<sup>2</sup>) of fluorescent: counterstained nuclei corresponding to (A) CD172a pan Mφ in normal (uninjured), sub-acute and chronic injured equine tendons; (B) CD14 (M1Mφ) and (C) CD206 (M2Mφ) expression in sub-acute and chronic injured tendons. (D) Log transformed ratio of immunopositive areas of M1M2Mφ from dual labeled CD14 and CD206 sections of sub-acute and chronically injured tendons. SAI= sub-acute injury (n=5), CI= chronic injury (n=5), N= normal tendon (n=5). All values represent median with maximum and minimum range. ***P<0.001, **P<0.01, *P<0.05.

Fig.2: Expression of FPR2/ALX is upregulated in sub-acute tendon injury and by pro-inflammatory mediators in vitro. (A) Box plot illustrating ratio of areas (μm<sup>2</sup>) of fluorescent: counterstained nuclei of the expression of the Lipoxin A<sub>4</sub> receptor (FPR2/ALX) in normal (N, n=5), sub-acute (SAI, n=5) and chronic injured (CI, n=5) equine tendons. Values represent median with maximum and minimum range. *P<0.05. (B) Effect of pro-inflammatory mediators on tendon FPR2/ALX expression in vitro, showing average expression (μm<sup>2</sup>) in SDFT explants from 3 normal horses. Explants were stimulated with 5ng ml<sup>-1</sup> IL-1β and 1.0μM PGE<sub>2</sub>, either alone or in combination and FPR2/ALX expression determined 0, 12, 24 and 72 hours after stimulation and compared to non-stimulated controls. **P<0.01, *P<0.05. Error bars denote SEM.