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# Osteoarthritis and Cartilage

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## Short Communication

### Immunolocalisation and expression of keratocan in tendon

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#### Introduction and summary

Tendon is a highly organised, dynamic tissue which functions to transmit tensile forces from muscle to bone. Type I collagen is the major component of the tendon extracellular matrix (ECM) and it is the ordered arrangement of collagen fibres which is critical in providing the high tensile strength of this tissue. Within the tendon, the endotenon (a thin reticular network of connective tissue) surrounds and binds the fibre bundles or “fascicles”, thus facilitating their physiological functioning<sup>1</sup>.

Importantly, the assembly (i.e., fibrillogenesis) of type I collagen in tendon is tightly regulated by small leucine-rich proteoglycans (SLRPs), and this process underpins the structural organisation of the tissue<sup>2</sup>. This is highlighted by genetic studies utilising mice deficient in one or two of the prominent SLRPs (i.e., decorin, biglycan, fibromodulin and lumican) which show clear structural alterations in their collagen fibrils, frequently resulting in impaired tendon function<sup>3</sup>. Within tendon, decorin is the most prevalent SLRP in regions which experience predominantly longitudinal/tensional forces, however, biglycan, fibromodulin and lumican have also been detected. In tendon fibrocartilage, which develops in regions compressed against bony pulleys and at tendon entheses, type II in addition to type I collagen is present, as well as increased levels of biglycan and other proteoglycans common to the articular cartilage phenotype<sup>4,5</sup>. Thus, regional differences in the expression of the various SLRPs in tendon may reflect such changes in the collagenous ECM. Similar to tendon, the ECM of cornea is composed of a highly ordered, fibrillar collagen network, and this is essential for the development and maintenance of corneal strength and transparency<sup>6</sup>. The keratan sulphate (KS)-containing SLRPs, lumican and keratocan, play a pivotal role in collagen fibrillogenesis in this tissue;

significantly, the absence of lumican leads to formation of cloudy corneas in homozygous knockout mice<sup>3</sup>, whilst those deficient in keratocan develop significantly larger diameter corneal collagen fibrils<sup>7</sup>.

Although a number of SLRPs have been identified and biochemically characterised in tendon, few studies have examined keratocan distribution and expression in this tissue. The present study was therefore conducted using bovine tendons from compressed/fibrocartilaginous and tensional regions to determine the immunolocalisation of keratocan in these distinct functional regions, using a new monoclonal antibody (mAb), KER-1, which recognises a protein epitope on the keratocan core protein. In addition, Western blot analysis was used to compare the expression of this SLRP in tissue extracts of tendon (fibrocartilaginous vs tensional), articular cartilage and cornea. The results of this study suggest that keratocan, previously thought to be ‘corneal-specific’<sup>8,9</sup>, is also abundant in tendon and is likely to play an important role in regulating the collagenous matrix of this tissue.

#### Methods, results and discussion

Bovine deep digital flexor tendons from compressed and tensional regions were dissected from the metacarpophalangeal joints of mature (18-month-old) animals<sup>10</sup>. Tensional samples were comprised of tissue located proximal to bifurcation of the tendon and compressed samples were composed of fibrocartilaginous tissue from the region of tendon distal to the bifurcation<sup>11</sup>. Full-depth articular cartilage was also dissected from the corresponding metacarpophalangeal joints. Corneas were harvested from the ocular globes of 18-month-old cattle.

For immunohistochemical analyses, tendon sections (cryosectioned at 10 µm in a transverse plane) were treated with chondroitinase ABC (0.4 U/ml), keratanase (0.4 U/ml), keratanase II (0.2 U/ml) and endo-β-galactosidase (0.002 U/ml) for 1 h at 37°C. Sections were subsequently blocked using 5% goat serum in 0.1% Tween/phosphate buffered saline (PBS) for 30 min and immunolabelled by indirect immunofluorescence using mAb KER-1 (1:10 dilution in 0.1% Tween/PBS and applied overnight at 4°C), which

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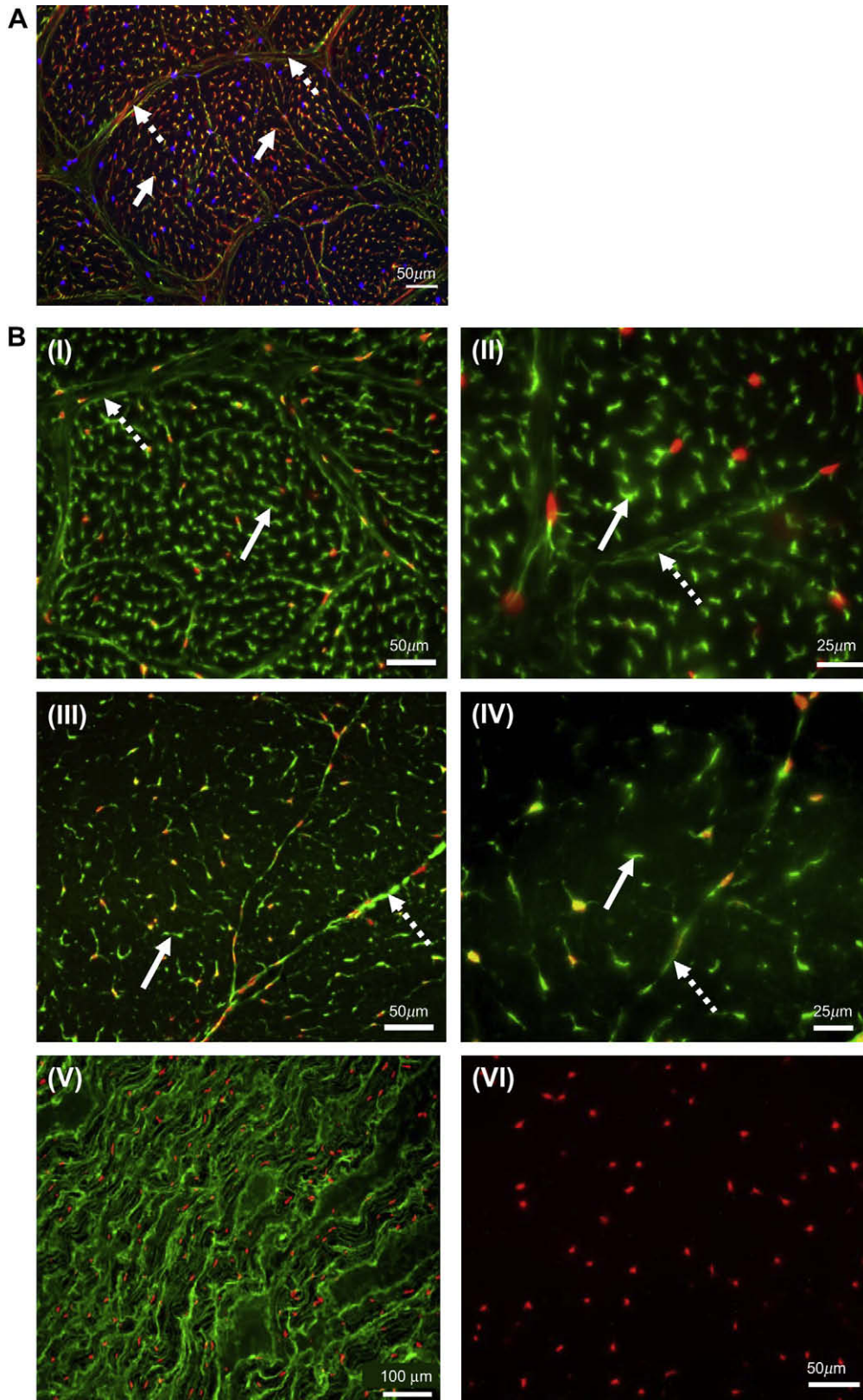


Fig. 1. Immunolocalisation of keratocan in mature bovine deep digital flexor tendon. (A) Transverse section of compressed tendon immunolabelled with mAb KER-1 (shown in red) which recognises the keratocan core protein and polyclonal antibody 70-XR90 (shown in green) which recognises type I collagen. Areas of co-localisation of keratocan/collagen I immunolabelling are shown in yellow. Cell nuclei are shown in blue. Positive immunolabelling of tendon collagen fibre bundles (solid arrows) and endotenon regions (dashed arrows) is indicated. (B) Transverse sections of compressed tendon (I, II), tensional tendon (III, IV) and cornea (V), immunolabelled with mAb KER-1, and counterstained with DAPI. Images are presented as red/green anaglyphs where positive immunolabelling is shown in green and cell nuclei in red (areas of co-localisation of keratocan immunolabelling with tendon cells are shown in yellow). Positive immunolabelling of tendon collagen fibre bundles (solid arrows) and endotenon regions (dashed arrows) is indicated (I–IV). Tendon sections with the primary antibody omitted showed no immunolabelling (VI).

recognises bovine keratocan core protein (the production of mAb KER-1 was carried out as previously described<sup>12</sup>). Visualisation was achieved using a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse secondary antibody (Dako, UK) and sections mounted in mounting-medium containing 4',6 diamidino-2-phenylindole (DAPI, Vectashield, Vector Laboratories). Immunohistochemical controls (as previously described<sup>13</sup>) showed no immunolabelling. Sections were viewed under epifluorescence using an Olympus BX61 microscope with appropriate filters for FITC and DAPI and equipped with digital image acquisition. Images were presented as red/green anaglyphs. In a series of separate experiments, dual-immunolabelling of tendon sections with antibodies to both type I collagen (rabbit polyclonal antibody 70-XR90, Fitzgerald Industries, USA) and keratocan (mAb KER-1) was carried out, showing their relative distributions in the tendon matrix, as a morphological reference. In addition, bovine corneas were cryosectioned and immunolabelled with mAb KER-1, as previously described<sup>12</sup>.

For sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS/PAGE) and Western blot analyses, tissue samples (tendon, cartilage or cornea) were finely diced and proteoglycans extracted twice at 4°C for 24 h with 15 volumes (w/v) of 4 M guanidine-HCl, 50 mM sodium acetate, pH 6.6, containing proteinases inhibitors<sup>5</sup>. The protein and sulphated glycosaminoglycan (GAG) content of extracts were subsequently determined using Bio-Rad DC protein assay (Bio-Rad Laboratories, UK) and dimethylmethylene blue (DMMB; Serva) colourimetric assay<sup>14</sup>, respectively. Samples were then treated with or without endo- $\beta$ -galactosidase (Seikagaku; 0.1 mU/10 mg GAG) in 0.2 M sodium acetate, 10 mM ethylenediaminetetra-acetate (EDTA), pH 5.8 for 1 h at 37°C, prior to separation by SDS/PAGE and Western blotting analysis (as previously described<sup>5</sup>), utilising mAb KER-1 (1:1000) which recognises the keratocan core protein<sup>12</sup>. Loading of samples onto the gels was standardised by loading on the basis of equivalent protein content (30  $\mu$ g of total protein per sample).

The results of immunohistochemical analyses are shown in Fig. 1. As expected, dual-immunolabelling studies revealed that type I collagen immunoreactive material was associated with the collagen fibre bundles and was also present in the endotenon, surrounding the collagen bundles. Keratocan immunolabelling showed a similar distribution, with strong co-localisation of type I collagen/keratocan immunostaining occurring around the collagen fibres bundles [Fig. 1(A)], compressed tendon only shown, however, similar results were obtained for tensional tendon]. Further investigation of the distribution of keratocan in compressed vs tensional tendon and cornea was performed [Fig. 1(B)]. Tissue sections of compressed tendon [Fig. 1(B)-I and II] showed a higher collagen bundle density compared with tensional tendon [Fig. 1(B)-III and IV] which may be attributed to the reduced collagen fibril diameter of the compressed region<sup>10</sup>. However, a similar pattern of keratocan immunoreactivity occurred in the two regions, with positive immunolabelling present in both the collagen fibre bundles and in the endotenon. In addition, KER-1 immunolabelling co-localised with the cells, which is likely to be associated with the synthesis/secretion of keratocan by the tenocytes and/or the modulation of cell function by this SLRP (in this regard, cell-associated keratocan has been shown to be present in the corneal epithelium<sup>12</sup> and various members of the SLRP family have been demonstrated to affect cellular responses<sup>2</sup>). In tissue sections of cornea, intense immunolabelling for keratocan occurred, and was associated with

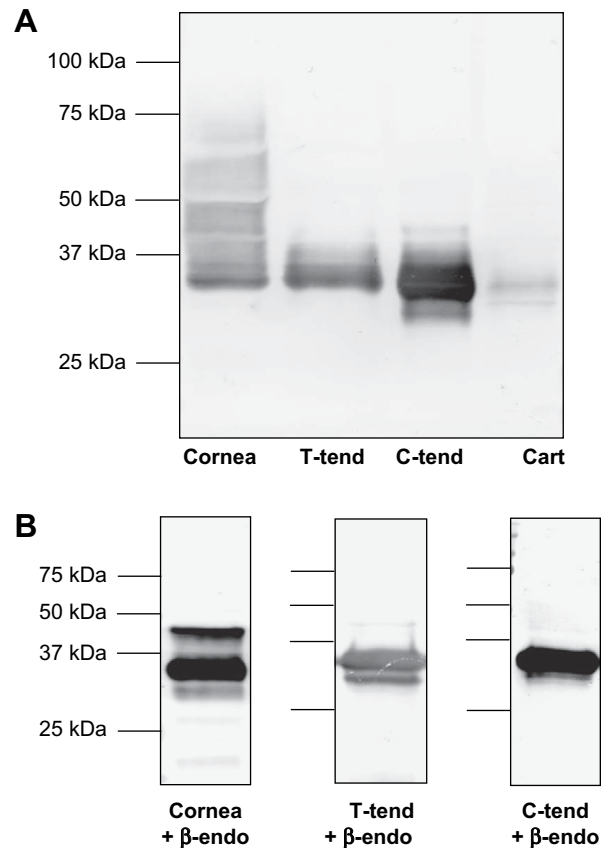


Fig. 2. Western blot analyses of tissue extracts of cornea, tensional tendon (T-tend), compressed tendon (C-tend) and articular cartilage (Cart), immunolocalised with mAb KER-1 which recognises the keratocan core protein. Samples were digested without (A) or with (B) endo- $\beta$ -galactosidase (+ $\beta$ -endo) to remove KS chains. Loading of samples onto the gels was standardised by loading on the basis of equivalent protein content of tissue extract (30  $\mu$ g).

the collagen fibres in the corneal stroma [Fig. 1(B)-V]. Negative controls showed no immunolabelling [Fig. 1(B)-VI].

Our data are consistent with previous studies which demonstrate that close and specific interactions occur between SLRPs and collagen fibrils in various connective tissues, including tendon and cornea<sup>2</sup>. Interestingly, the immunolocalisation pattern of keratocan (Fig. 1) is similar to that of decorin within mouse tendon, with positive immunostaining occurring in both tendon collagen bundles and surrounding connective tissues (endotenon regions)<sup>15</sup>. This distribution was distinct to that reported for both fibromodulin and lumican, where staining was localised to either the collagen fibre bundles or the surrounding tissues, respectively, in the wild type animal<sup>15</sup>. As collagen fibrils are important constituents of the endotenon<sup>1</sup>, it is therefore likely that certain SLRPs (e.g., keratocan and lumican) are involved in the regulation of collagenous components within this loose matrix.

The results of Western blot analyses are shown in Fig. 2. Differences were apparent in the relative abundance of keratocan to total protein in the various tissues examined, with cornea and compressed tendon exhibiting the most intense immunostaining (consistent with the high proteoglycan content of tendon fibrocartilage<sup>4</sup>), compared with little immunoreactivity in cartilage extracts [Fig. 2(A)]. In addition, variations were apparent in post-translational modifications (i.e., GAG substitutions) in keratocan derived from these

tissues. For example, keratocan immunoreactivity in cornea was detected as heterogeneous bands, ranging in molecular mass from ~35 kDa to ~75 kDa, indicative of a glycosylated core protein, whereas keratocan derived from tendon and cartilage exhibited little glycosylation, with immunopositive bands ranging from ~35 kDa to ~46 kDa. In order to confirm this, tissue samples were digested with endo- $\beta$ -galactosidase (which specifically hydrolyses the  $\beta$ -galactosidic linkages of non-sulphated galactosyl residues in KS and has weaker specificity for mono-sulphated galactosyl residues); this resulted in a reduction in molecular mass of the corneal keratocan following Western blotting, in contrast to tendon [Fig. 2(B)] and cartilage samples (data not shown), which showed little change in the range of immunopositive staining. Taken together, these data indicate that the core protein of adult corneal keratocan is highly glycosylated, whereas in tendon and cartilage, the KS-GAGs in this SLRP are poorly sulphated, non-sulphated or not present. However, it is important to note that certain KS-SLRPs (e.g., fibromodulin) have been shown to be poorly substituted with KS in the adult, but to possess KS chains in the juvenile<sup>16</sup>, raising the possibility that age-related changes may also occur in keratocan glycosylation. Interestingly, keratocan with minimally sulphated KS chains (such as that present in tendon, as well as other non-corneal tissues) has the potential to affect cellular responses<sup>17</sup>, in a similar fashion to other members of the SLRP family<sup>2</sup>.

In conclusion, the results of this study demonstrate that keratocan, previously thought to be 'corneal-specific' in adult tissues, is abundant in tendon and, as such, is likely to play an important part in regulating its collagenous matrix, a process vital to the correct physiological functioning of this tissue.

### Conflict of interest

The authors declare no conflict of interest.

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