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Immunohistochemical distribution of type I, II and III collagens in the rabbit supraspinatus tendon insertion

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ABSTRACT

The collagen fibres in the area of attachment of the supraspinatus tendon to bone were studied immunohistochemically in 12 mature female New Zealand white rabbits. The labelling of type I collagen was uniformly prominent in the bone as well as in the fascicles of the tendon proper but inconspicuously scattered in the unmineralised and mineralised zones of the fibrocartilage. Type II collagen, not detected in the tendon proper, was widespread in both zones of the fibrocartilage. Type III collagen, on the other hand, appeared to be confined mainly to the zone of unmineralised fibrocartilage, in addition to its presence in the endotenon of the tendon proper. The region of the tidemark failed to show immunostaining for any of the collagen fibre types. In conclusion, this study demonstrates that, although all the principal fibrous collagen types are constituents of the supraspinatus tendon at its attachment site, the distribution pattern of immunolabelling varies from zone to zone.

INTRODUCTION

A characteristic feature of the insertion of tendons into bone is the presence of fibrocartilage (Woo et al. 1988; Benjamin & Evans, 1990). Dalgo-Saburoff (1929) was the first to describe the attachment site of a tendon to bone by dividing the area into 4 zones: the zone of unmineralised fibrocartilage that is closest to the tendon proper; the tidemark, a prominent basophilic line which indicates the outer limit of calcification, and which separates the zone of unmineralised fibrocartilage from the next zone of mineralised fibrocartilage and, finally, the bone. Cooper & Misol (1970) divided the area of attachment into tendon proper, unmineralised fibrocartilage, mineralised fibrocartilage and bone, and gave a detailed description of cells and matrix that comprise the fibrocartilage. Many of the human tendons with epiphyseal and metaphyseal attachment to long bone, have been shown to have similar structural divisions (Benjamin et al. 1986).

Biochemical studies on flexor tendons have determined that type I collagen constitutes in excess of 95% of the total collagen component of normal adult tendons, and the remaining collagens are of types III and V (Jimenez et al. 1978; Cetta et al. 1982; Gelbermann et al. 1988). However, it has been clearly demonstrated by immunohistochemical stains that type II collagen is abundantly present in the fibrocartilage of the attachment zone of a tendon (Benjamin et al. 1991; Ralphs et al. 1992; Rufai et al. 1992).

In the course of our studies on the rotator cuff, we examined the principal collagen fibre types at the zone of insertion of the supraspinatus tendon in rabbits with immunohistochemical techniques using monoclonal antibodies. We found in the fibrocartilage that in addition to the presence of type II collagen in both the unmineralised and the mineralised parts, the labelling for type III collagen was strong in the unmineralised part. In contrast, immunoreactivity for collagen type I in either part of the fibrocartilage was sparse.

MATERIALS AND METHODS

Specimens of the supraspinatus tendon with its insertion into the greater tuberosity of the humeral head were removed from 12 mature female New

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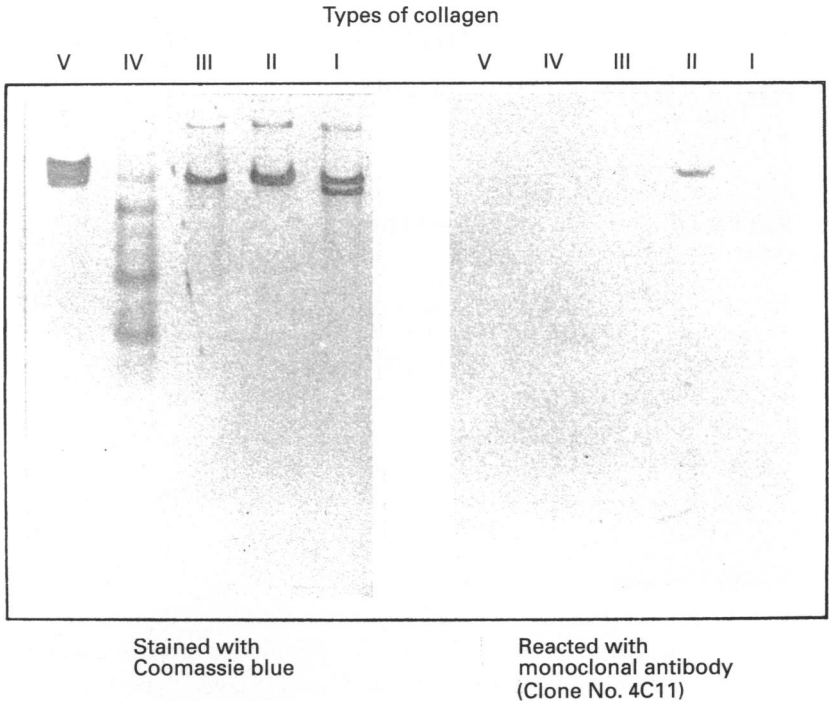

Fig. 1. Immunoblot analysis of human type II collagen.

Zealand white rabbits (*Lepus brachyrus*) weighing between 3.8 kg and 5.2 kg (average weight 4.2 kg). The animals were first euthanised with 5 ml of intravenous pentobarbital (Euthanyl, 240 mg/ml, CDMV Inc. St-Hyacinthe, Quebec, Canada).

Specimens were fixed in 10% neutral buffered formalin for 3–4 d, and then decalcified in 10% ethylenediamine tetra-acetic acid (EDTA) for a period of 4–5 wk. The completion of decalcification was confirmed by radiological examination. For routine histological examination the paraffin-embedded tissue was sectioned along the long axis of the tendon in the frontal plane. Sections (5 μm) were stained with haematoxylin and eosin and with toluidine blue.

For immunohistochemical examination, the following methods were performed in sequence: (1) preparation of monoclonal antibodies against collagen types; (2) immunohistochemical procedures; (3) counterstaining with haematoxylin.

Preparation of monoclonal antibodies

The monoclonal antibodies to collagen I, II and III were prepared in the laboratory of one of the authors (A.O.) of this study. The details of the preparation against type I and III collagens have been reported previously (Muragaki, 1985; Sakakibara et al. 1986; Matsumoto, 1988). We describe here the preparation of monoclonal antibody to type II collagen because it has not been reported before.

Human type II collagen was extracted from costal cartilage and purified by the method of Rhodes & Miller (1978). BALB/c mice were immunised by subcutaneous injection of 100 μg of human type II collagen emulsified with an equal volume of complete Freund's adjuvant. Three weeks after the 6th injection, spleen cells were hybridised with myeloma cell line (P3-X-63-Ag8-LUI) by the method of Köhler & Milstein (1975). The hybridoma was selected in the hypoxanthine adenine thymidine (HAT) medium. Positive hybrids were screened by an enzyme-linked immunosorbent assay (ELISA) using a type II collagen-coated microplate. Hybrids were cloned by the limiting dilution method. Monoclonal antibodies thus produced were specific for type II collagen. No cross reaction was noted for any of types I, III, IV and VI collagen. Specificity of the antibody was further determined by an immunoblot.

The α1(II) chain of type II collagen was separated on SDS polyacrylamide gel (7%) according to the method of Laemmli (1970). The protein bands were electroblotted on nitrocellulose membrane and reacted with antihuman type II collagen monoclonal antibody. Immunoreaction was visualised by avidin-biotin complex method. Specific reaction was demonstrated on the protein-band of α1(II) chain (Fig. 1).

Immunohistochemical procedure

The paraffin-embedded tissues were serially sectioned at 5 μm and placed on slides coated with 2% 3-aminopropyltriethoxysilane which prevents detachment of sections (Rentrop et al. 1986). After deparaffinisation with xylene, sections were rehydrated in graded ethanol and treated with 0.25% trypsin (Gibco, Burlington, ON, Canada) dissolved in tris-buffered saline (TBS) with a pH of 7.6 for 30 min at 37 °C. A final wash with TBS was done to stop all enzyme activity. Sections were then treated with testicular hyaluronidase (1.45 IU/ml; type I-S, Sigma Chemical Company, St Louis, MO, USA) and chondroitinase ABC (0.25 IU/ml; Sigma Chemical Company) for 30 min at 37 °C prior to incubation with primary antibodies.

Following Yulis & Lederis (1987), all primary antibodies were diluted with TBS containing 0.1% Triton X-100 (Sigma Chemical Company) and 0.7% carrageenan (Sigma Chemical Company). The antiserum to type I collagen was used at 1:100 dilution (10 μg/ml protein concentration) and those to type II and III were at 1:10000 dilution (10 ng/ml protein concentration). The incubation time was overnight at room temperature. The sections were then incubated with rabbit antimouse Ig (Dako, Mississauga, ON, Canada) as the secondary antibody in dilutions of 1:25 for 60 min. After washes with TBS, mouse peroxidase-antiperoxidase (PAP) complex (Dako) in a dilution of 1:250 was applied to the sections for 60 min. Finally, the sections were soaked in 0.02% solution of diaminobenzidine tetrachloride in TBS containing 0.05% H_2O_2 for 20 min for colourisation.

The control studies included substitution of the primary antibody with the antibody diluent and absorption tests using excess amount of respective collagen.

Counterstaining with haematoxylin

At the end of the immunohistochemical procedures, the slides were counterstained with haematoxylin which improved demonstration of cells.

RESULTS

With haematoxylin and eosin staining, the area of insertion of the rabbit supraspinatus tendon showed the characteristic zonal divisions into unmineralised fibrocartilage, the tidemark, mineralised fibrocartilage and bone. The zone of unmineralised fibrocartilage was proportionally larger than its mineralised counterpart, and the central region of the fibrocartilage appeared to extend further into the tendon proper than the outer regions. The cells in the unmineralised fibrocartilage were arranged in short rows and resembled chondrocytes with a pericellular halo surrounded by a densely stained matrix (Fig. 2). The packed fibres were oriented along the long axis of the tendon, perpendicularly to the tidemark. The zone was devoid of vascular channels and there were no clearly discernible spaces representing endotenon.

The tidemark, or the blue line, which denotes the calcification front of the mineralised fibrocartilage, had a wavy configuration. Duplication of the tidemark was common (Fig. 2). Distal to the tidemark, the mineralised fibrocartilage occupied a narrow area. Here the cells were not arranged in rows as seen in the unmineralised part but were relatively small in number and randomly dispersed.

Following combined hyaluronidase and chondroitinase digestion, the immunohistochemical labelling was strong for types II and III but demonstrably weak

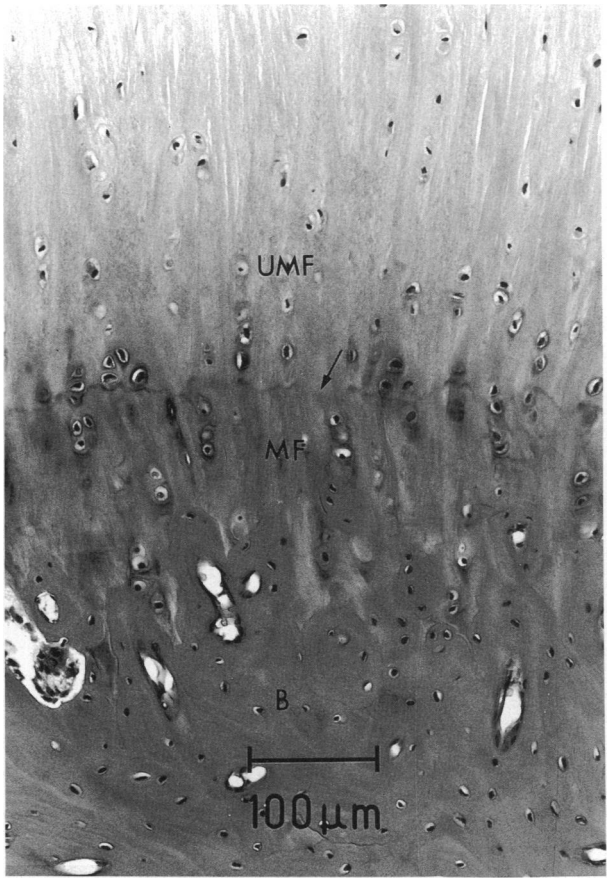

Fig. 2. Bony insertion of the supraspinatus tendon: zone 1, unmineralised fibrocartilage (UMF); zone 2, tide mark (arrow); zone 3, mineralised fibrocartilage (MF); zone 4, bone (B). Haematoxylin and eosin stain.

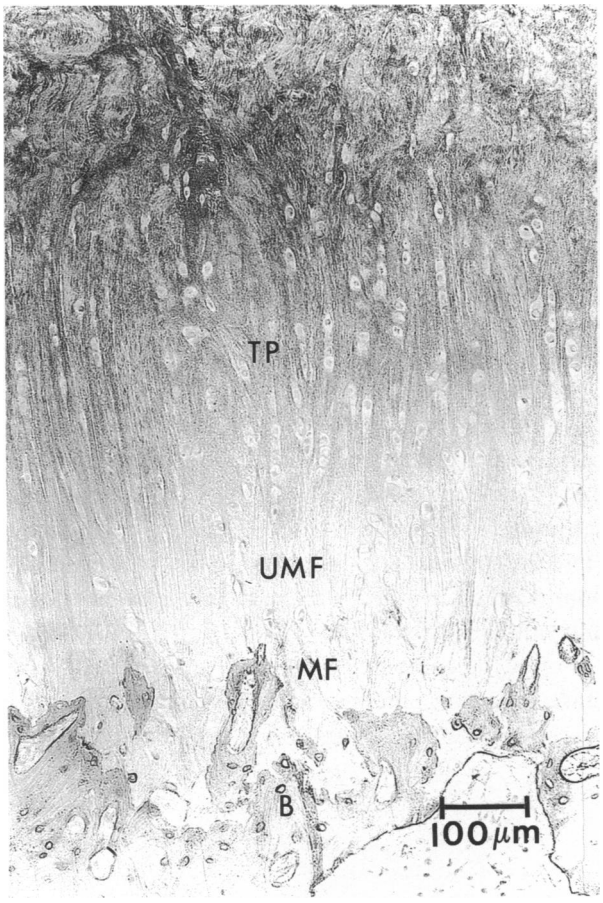

Fig. 3. Positive immunohistochemical staining for type I collagen in bone (B) and the tendon proper (TP). UMF, unmineralised fibrocartilage; MF, mineralised fibrocartilage.

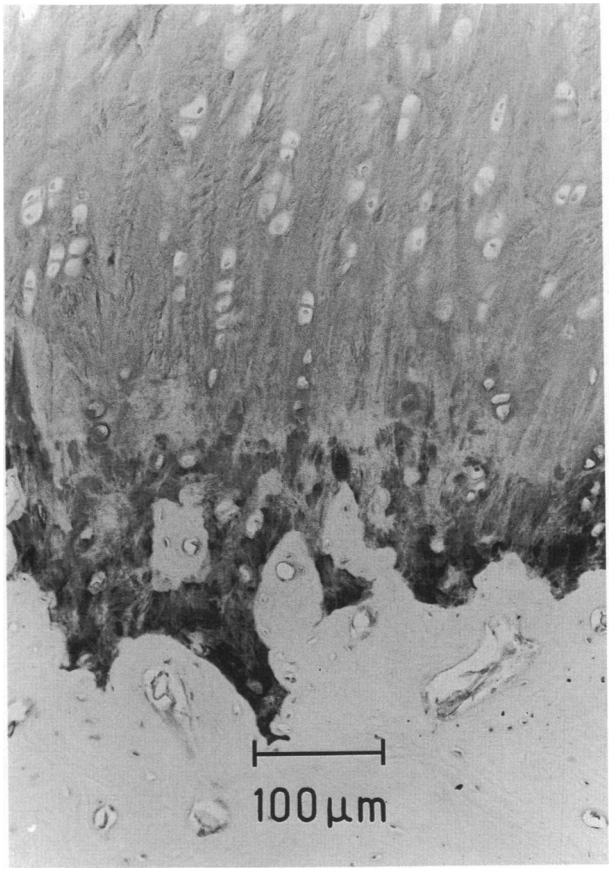

Fig. 4. Positive immunohistochemical staining for type II collagen in the unmineralised fibrocartilage as well as in the mineralised fibrocartilage; the staining is more intense in the latter. The intervening tidemark and bone are unstained.

for type I, although the antibody of the latter was diluted much less than the antibodies for the other 2 types. Only a few scattered fibres of type I collagen could be seen in either part of the fibrocartilage (Fig. 3). The bone and the fascicles in the tendon proper, however, showed uniformly dispersed labelling for type I.

Immunostaining for type II collagen was prominent in the unmineralised as well as in the mineralised parts of the fibrocartilage (Fig. 4). The staining was band-like along the long axis of the tendon in the unmineralised part, but somewhat less organised in the mineralised part. The labelling did not extend to the bone nor to the tendon proper.

Immunolabelling for type III collagen was conspicuous in the unmineralised fibrocartilage, especially in the vicinity of chondrocytes (Fig. 5). It was only occasionally seen in the mineralised fibrocartilage. In the tendon proper, however, the endotenon showed positive reactivity for type III, particularly around the blood vessels. The fascicles of the tendon proper and the bone were unstained.

The tidemark, in general, showed a relative lack of labelling by any of the 3 collagen types. The control slides showed uniformly negative results.

DISCUSSION

The present histochemical study on the bony insertion of the rabbit supraspinatus tendon showed that the zones of the attachment site had different patterns of immunoreactivity to monoclonal antibodies against types I, II and III collagen. Two aspects of the immunolabelling pattern, however, remain unexplained with the present scope of the study: the tidemark was generally resistant to immunolabelling for any of the 3 fibrous collagen types, and staining for type I collagen was quite sparse in the 2 zones of the fibrocartilage.

The tidemark, which represents the outer limit of the mineralised fibrocartilage, often tends to be discontinuous, and it was no exception in the rabbit supraspinatus tendon. The tidemark, studied by electron microscopy in an articular cartilage, has been

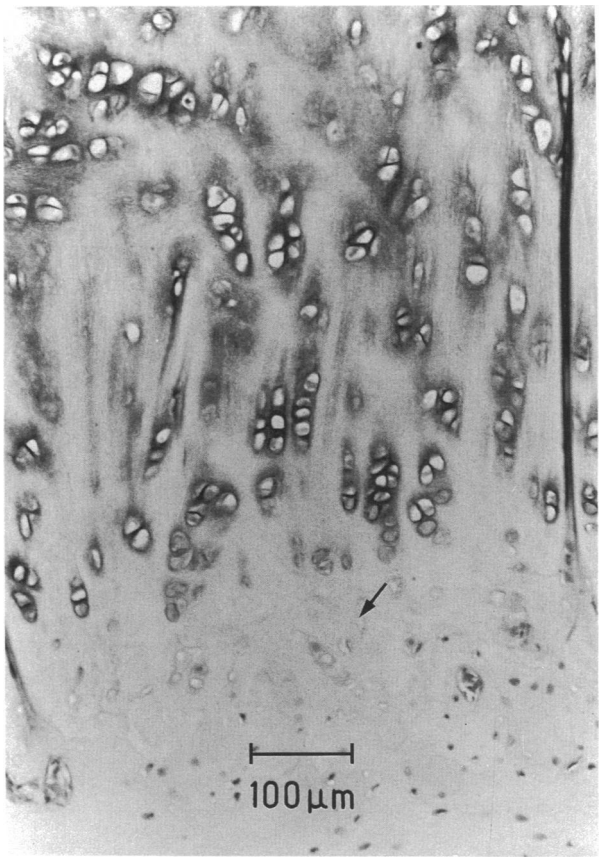

Fig. 5. Positive immunohistochemical staining for type III collagen in close relation to chondrocytes in the unmineralised fibrocartilage. The tidemark (arrow), mineralised fibrocartilage and bone are unstained.

shown to be composed of densely packed, randomly oriented collagen fibrils of varying diameters that are continuous with those of the unmineralised and mineralised fibrocartilage (Redler et al. 1975). In the present study, the failure to label any of the 3 types of collagen in the tidemark could not have been due to 'masking' of the fibres by mineral deposits of calcium or the ground substance because the tissue was appropriately decalcified and also pretreated with hyaluronidase and chondroitinase before immunolabelling.

The sparsity of immunolabelling for type I collagen in the fibrocartilage could have been due to several reasons. The specificity of the monoclonal antibody was not, however, in doubt since the bone as well as the fascicles in the tendon proper stained uniformly. A relative lack of accessibility of the type I collagen to immunostaining might be a factor. Recent studies have shown that collagen fibrils of many tissues are 'heterotypic' structures in which fibril-forming collagens are coassembled with one another in different combinations (Linsenmayer, 1991). It is possible then, that most of the type I collagen fibres are overridden by other collagen types. We found strong immunolabelling for type III collagen fibres which tend to be predominantly located at the fibril surface (Burgeson & Nimni, 1992), and it thus could prevent immunolabelling for type I. Finally, there might very well be a paucity of type I collagen fibres at the attachment site, unless biochemical determinations of this particular area prove otherwise.

The presence of type III collagen at the attachment site has not been reported before. It was significant that type III collagen was principally confined to the unmineralised part of the fibrocartilage which is much more compliant than the relatively rigid mineralised part. The presence of type III collagen has been directly correlated with tissue extensibility (Burgeson & Nimni, 1992), hence the importance of the presence of type III fibres at the site where the supraspinatus tendon bends over the convexity of the humeral head for its insertion into the greater tuberosity.

We conclude, that immunohistochemical studies are helpful in elucidating certain aspects of structural complexities of the attachment site of the supraspinatus tendon which is frequently involved in rotator cuff injuries. Biochemical quantitation of the fibrous collagen types in the fibrocartilage including the tidemark is lacking (Woo et al. 1988). Furthermore, the concentration of type III in tissues is often underestimated in biochemical examination because of its insolubility (Cheung et al. 1990). Our study clearly identifies the significant presence of III collagen type along with type II at the attachment site of the rabbit supraspinatus tendon.

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REFERENCES

Benjamin M, Evans EJ (1990) Fibrocartilage. *Journal of Anatomy* **171**, 1–15.

Benjamin M, Evans EJ, Copp L (1986) The histology of tendon attachments to bone in man. *Journal of Anatomy* **149**, 89–100.

Benjamin M, Tyers RNS, Ralphs JR (1991) Age-related changes in tendon fibrocartilage. *Journal of Anatomy* **179**, 127–136.

Burgeson RE, Nimni ME (1992) Collagen types. Molecular structure and tissue distribution. *Clinical Orthopaedics and Related Research* **282**, 250–272.

Cetta G, Tenni R, Zanaboui G (1982) Biochemical and morphological modifications in rabbit achilles tendon during maturation and ageing. *Biochemical Journal* **204**, 61–67.

Cheung DT, Benya PD, Perelman N, DiCesare PE, Nimni ME

(1990) A highly specific and quantitative method for determining type III/I collagen ratios in tissues. *Matrix* **10**, 164–173.

COOPER R, MISOL S (1970) Tendon and ligament insertion. *Journal of Bone and Joint Surgery* **52A**, 1–20.

DOLGO-SABUROFF B (1929) Ueber Ursprung and Insertion der Skelettmuskeln. *Anatomischer Anzeiger* **68**, 80–87.

EVANS EJ, BENJAMIN M, PEMBERTON DJ (1990) Fibrocartilage in the attachment zones of the quadriceps tendon and patellar ligament of man. *Journal of Anatomy* **171**, 155–162.

GELBERMAN R, GOLDBERG V, AN K-N, BANES A (1988) Tendon. In *Injury and Repair of the Musculoskeletal Soft Tissues* (ed. S. L-Y. Woo & J. A. Buckwalter), pp. 5–40. Illinois: American Academy of Orthopaedic Surgeons.

JIMENEZ SA, YANKOWSKI R, BASHEY RI (1978) Identification of two new collagen-chains in extracts of lathyritic chick embryo tendons. *Biochemical and Biophysical Research Communications* **81**, 1298–1306.

KÖHLER G, MILSTEIN C (1975) Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature* **256**, 495–497.

LAEMMLI UK (1970) Cleavage of structural proteins during the assembly of the head of bacterophage T4. *Nature* (London) **227**: 680–685.

LINSENMAYER TF (1991) Collagen. In *Cell Biology of Extracellular Matrix* (ed. E. D. Hay), 2nd edn, pp. 7–44, New York: Plenum Press.

MATSUMOTO E (1988) Foreknowledge of liver fibrosis: development of immunoassay system for serum collagen peptides with monoclonal antibody. *Journal of Wakayama Medical Society* **39**, 87–106.

MURAGAKI Y (1985) Experimental study on alteration of collagen metabolism in liver fibrosis. *Journal of Wakayama Medical Society* **36**, 195–208.

RALPHS JR, TYERS RNS, BENJAMIN M (1992) Development of functionally distinct fibrocartilages at two sites in the quadriceps tendon of the rat: the suprapatella and the attachment to the patella. *Anatomy and Embryology* **185**, 181–187.

REDLER I, MOW VC, ZIMNY ML, MANSELL J (1975) The ultrastructure and biomechanical significance of the tide mark of articular cartilage. *Clinical Orthopaedics and Related Research* **112**, 357–362.

RENTROP M, KNAPP B, WINTER H, SCHWEIZER J (1986) Aminoalkylsilane-treated glass slides as support for in situ hybridization of keratin cDNA to frozen tissue sections under varying fixation and pretreatment conditions. *Histochemical Journal* **18**, 271–276.

RHODES RK, MILLER EJ (1978) Physicochemical characterization and molecular organization of the collagen A and B chains. *Biochemistry* **17**, 3442–3448.

RUFAI A, BENJAMIN M, RALPHS JR (1992) Development and aging of phenotypically distinct fibrocartilage associated with the rat Achilles tendon. *Anatomy and Embryology* **186**, 611–618.

SAKAKIBARA K, OOSHIMA A, IGARASHI S, SAKAKIBARA J (1986) Immunolocalization of type III collagen and procollagen in cirrhotic human liver using monoclonal antibodies. *Virchows Archiv A* **409**, 37–46.

WOO S, MAYNARD J, BUTLER D, LYON R, TORZILLI P, AKESON W, et al. (1988) Ligament, tendon and joint capsule insertions to bone. In *Injury and Repair of the Musculoskeletal Soft Tissues* (ed. S. L-Y. Woo & J. A. Buckwalter), pp. 133–166, Illinois: American Academy of Orthopaedic Surgeons.

YULIS CR, LEDERIS K (1987) Co-localization of the immunoreactivities of corticotropin-releasing factor and arginine vasotocin in the brain and pituitary system of the teleost Catostomus commersoni. *Cell and Tissue Research* **247**, 267–273.