Cell Tissue Res (2006) 325: 315–324
DOI 10.1007/s00441-006-0161-0

REGULAR ARTICLE

Hernandes F. Carvalho · Sérgio L. Felisbino · Douglas R. Keene · Kathryn G. Vogel

Identification, content, and distribution of type VI collagen in bovine tendons

Received: 7 September 2005 / Accepted: 5 January 2006 / Published online: 28 March 2006

Abstract Tendon composition changes according to differentiation, mechanical load, and aging. In this study, we attempted to identify, localize, and quantify type VI collagen in bovine tendons. Type VI collagen was identified by the electrophoretic behavior of the alpha chains and Western blotting, and by rotary shadowing. Type VI collagen was extracted from powdered tendon with three sequential 24-h extractions with 4 M guanidine-HCl. The amount of type VI collagen was determined by enzyme-linked immunosorbent assay for purely tensional areas and for the compressive fibrocartilage regions of the deep flexor tendon of the digits, for the corresponding fetal and calf tendons, and for the extensor digital tendon. The distal fibrocartilaginous region of the adult tendon was richer in type VI collagen than the tensional area, reaching as much as 3.3 mg/g (0.33%) of the wet weight. Calf tendons showed an accumulation of type VI at the fibrocartilage site. Immunocytochemistry demonstrated that type VI collagen was evenly distributed in the tensional areas of tendons but was highly concentrated around the fibrochondrocytes in the fibrocartilages. The results demonstrate that tendons are variable with regard to the presence and distribution of type VI collagen. The early accumulation of type VI collagen in the region of calf tendon that will become fibrocartilage in the adult suggests that it is a good marker of fibrocartilage differentiation. Furthermore, the distribution of type VI collagen in tendon fibrocartilage indicates that it organizes the pericellular environment and may represent a survival factor for these cells.

Keywords Compressive load · Tendon · Tendon fibrocartilage · Type VI collagen · Bovine

This work was supported with funds from NIH/USA (to K.G.V.) and from FAPESP/São Paulo State, Brazil (to H.F.C.).

H. F. Carvalho (✉)
Department of Cell Biology, Institute of Biology,
State University of Campinas,
Campinas SP, Brazil
e-mail: hern@unicamp.br

S. L. Felisbino
Department of Morphology, State University of São Paulo,
Botucatu SP, Brazil

D. R. Keene
Shriners Hospital for Children Microscopy Unit,
Portland, OR 97201, USA

K. G. Vogel
Department of Biology, University of New Mexico,
Albuquerque, NM 87131, USA

Introduction

One variant of the basic tendon structure is found within the classical tendons of various species in areas subjected to compressive load (Gillard et al. 1979; Merrilees and Flint 1980; Okuda et al. 1987; Vogel and Koob 1989; Rufai et al. 1992; Carvalho and Vidal 1994a,b; Covizi et al. 2001). All these tendons exhibit, in their compressed regions, compositional and structural characteristics of fibrocartilages, including increased content of glycosaminoglycans/proteoglycans, some type II collagen, basket-weave-like arrangements of collagen fibers, and fibrochondrocytes or fibrochondrocyte-like cells. The bovine deep flexor tendon (DFT) has long been used as a model of tendon response to compressive load (Evanko and Vogel 1990), as it develops a fibrocartilaginous pad in the area in which it wraps around a joint. Many aspects of the structure and composition of this area have been described (Vogel and Koob 1989), as have important features of its metabolism (Evanko and Vogel 1990, 1993; Koob et al. 1992; Robbins and Vogel 1994; Vogel 1996). The bulk of the results show a coherent response to compressive load with compositional changes toward the fibrocartilage phenotype.

Type VI collagen has a ubiquitous distribution in tissues (Timpl and Chu 1994), being early described in tendons and in the secreted medium of fibroblasts. Given its morphology and distribution in tendons, it has been attributed an integrative function, organizing collagen fibers and linking them to the cells (Bruns et al. 1986). Type VI collagen has been found in the osseous attachment

to the myotendinous junction of the masseter tendon (Senga et al. 1995), and spatial interactions of type VI collagen, small proteoglycans, and collagen fibrils have been observed in tendons (Watanabe et al. 1997). Nurminskaya and Birk (1998) have shown that type VI collagen is expressed after the fibrillogenesis phase. This pattern of deposition is consistent with the exclusion of type VI collagen from the tendon mid-substance but its accumulation around the cells (Felisbino and Carvalho 1999).

Type VI collagen has been previously described in the tensile area of the bovine DFT (Vogel and Meyers 1999) and in tendon fibrocartilage (Vogel and Peters 2005). In the current study, we identify, isolate, and compare the amount of type VI collagen in various tendon regions, aiming at establishing correlations with different mechanical loads. In addition, we have investigated the variations in content and distribution during the development of the bovine tendon fibrocartilage. To accomplish this task, we have used a combination of biochemical, immunochemical, and microscopic analyses. The results obtained here demonstrate (1) the presence of type VI collagen in various bovine tendons and tendon regions of bovines, (2) the progressive increase in the amount of type VI collagen with respect to tendon maturation, (3) the accumulation of type VI collagen in the fibrocartilaginous areas, and (4) the concentrated location of type VI collagen in the pericellular matrix of fibrochondrocytes.

Materials and methods

Animals and tissue sampling

Bovine tendons were obtained from a slaughterhouse near Albuquerque, N.M., USA or from the Veterinary School-UNESP in Botucatu SP, Brazil. Samples were obtained within 3 h after death. Some specimens were frozen at −20°C prior to use. Those destined for immunocytochemistry or transmission electron microscopy were either immediately frozen in liquid nitrogen or fixed according to the specific protocol. Animals were divided into three age groups: fetal animals corresponded to animals from the 7th month of pregnancy to term; calves were animals up to 6 months old; adult animals were between 1 and 2 years old.

The flexor digitorum profundus (DFT) of the hind leg of each animal was taken and divided into two regions: proximal (purely tensile loads) and distal (subjected to compressive loads). In adult tendons, the surface region receiving direct loading as the tissue passes under the bone was used, as described by Vogel et al. (1986). The adult digital extensor tendon was included in some of the analyses for comparison, because it receives lower and purely tensile loading. The tissue was frozen in liquid nitrogen, powdered, and stored at −70°C.

Identification of type VI collagen

Type VI collagen was extracted from the distal region of adult bovine DFT. Powered tendon (1 g) was extracted for 24 h with gentle stirring in 25 volumes of 4 M guanidine-HCL (Gn-HCl) in 50 mM acetate buffer, pH 5.8, containing protease inhibitors, at 4°C. The extract was cleared by centrifugation, and 100 μl were precipitated with 9 volumes of cold ethanol. The dried precipitate was dissolved in sample buffer in the presence or absence of dithiothreitol and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4%–20% acrylamide gradient gels. After electrophoresis, the proteins were transferred to nitrocellulose membranes and assayed by Western blotting with two polyclonal antibodies against the α3(VI) subunit (antibody nos. 279 and 521), kindly provided by Dr. E. Engvall (La Jolla Calif., USA).

Extraction and identification of type VI collagen in tendons

Most of the type VI collagen was extracted from the various tendons after three rounds of a 24-h extraction in cold Gn-HCL. Treatment with dithiothreitol has been shown to extract a residual fraction of type VI collagen from the tensile region of bovine tendons (Vogel and Meyers 1999), but this corresponds to less then 5% of the amount obtained with the Gn-HCl extraction; this type of extraction was thus omitted in the present case. Extracts were prepared from the proximal and distal regions of the fetal, calf, and adult flexor tendons. The adult extensor digital tendon was also assessed.

Isolation of tendon type VI collagen

The Gn-HCL extract was cleared by centrifugation and concentrated by ultrafiltration, under nitrogen pressure, in an Amicon unit. The concentrated extract was loaded onto a Sepharose CL4B column (2.5×100 cm) equilibrated in 4 M Gn-HCl. The column was then eluted with 4 M Gn-HCl, and 10 ml fractions were assessed for protein content (absorbance at 280 nm) and for sulfated glycosaminoglycans by the dimethylmethylene blue (DMMB) method (Farndale et al. 1986). The void volume fractions were pooled and dialysed against 2 M urea in 50 mM TRIS/HCl, pH 8.3 (lower pH values resulted in aggregate formation even in the presence of 7 M urea). The sample was then applied to a DEAE-Sephacel column (2×10 cm) to separate type VI collagen from large proteoglycans. Type VI collagen was eluted from the column with low salt (0.2 M NaCl), whereas the large proteoglycans required higher molarity (0.8–1.0 M NaCl) to be eluted. The material eluted with low salt was pooled and analysed by SDS-PAGE and

rotary shadowing, as described by Morris et al. (1986). The material was then lyophilized and weighed to be used as a standard for the enzyme-linked immunosorbent assay (ELISA).

Analysis by ELISA

An ELISA method for the quantification of type VI collagen in bovine tendons was developed. To cover the 96-well plates, it was necessary to dilute the samples, especially the first Gn-HCl extract, which had a high protein content that masked the amount of type VI. We prepared serial dilutions from these extracts to determine the optimal concentration of the extract that would result in the best determination of type VI collagen. Dilutions varied from 1:4 to 1:24. Purified type VI collagen was included as a standard. Type I collagen and aggrecan purified from the distal region of the bovine flexor tendon were used as controls in this assay.

The wells were covered with optimally diluted Gn-HCl extracts overnight and sequentially assayed for the amount of type VI collagen by using the no. 279 antibody diluted 1:5,000, a peroxidase-conjugated goat anti-rabbit Igs (Sigma, Saint Louis, Mo., USA) and orthophenylene diamine as the peroxidase substrate. Measurements were made in triplicate, and the experiments were repeated three times for the adult tendons and twice for the fetal and calf tendons. The results are presented as the total amount of type VI collagen in all three sequential extractions of each sample. One-way analysis of variance (ANOVA) was used to compare the measurements obtained after each treatment with those of the controls.

Immunocytochemistry

Tendons were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 12 h. Frozen sections (5 μm thick) were cut, allowed to dry, fixed with acetone for 10 min, air-dried once again, rehydrated in PBS, and subjected to immunocytochemistry by using an antibody against the type VI collagen α_3 chain (no. 279). Better results were obtained when the sections were first treated with 1% bovine testis hyaluronidase in PBS for 30 min at room temperature. After enzyme digestion, sections were treated with 3% hydrogen peroxide in water for blocking endogenous peroxidases and with 3% bovine serum albumen in PBS to block non-specific protein interactions.

En bloc ATP treatment and transmission electron microscopy

Tissue fragments were incubated in 20 mM ATP in PBS for 1 h at 37°C (Bruns et al. 1986; Felisbino and Carvalho 1999). Controls were incubated in PBS without ATP. ATP-treated samples and controls were fixed in 4% glutaraldehyde and 0.25% tannic acid in Millonig´s buffer, post-fixed in osmium tetroxide, and embedded in Epon 812, after being dehydrated in a graded acetone series. Ultrathin sections were stained with uranyl acetate and lead citrate and examined by using a Philips CM100 electron microscope.

Results

Identification of type VI in bovine tendons

The putative α chains of type VI collagen were identified by their electrophoretic behavior under reducing conditions, by their apparent molecular mass (Fig. 1a), and by Western blotting. The two antisera (nos. 279 and 521) were

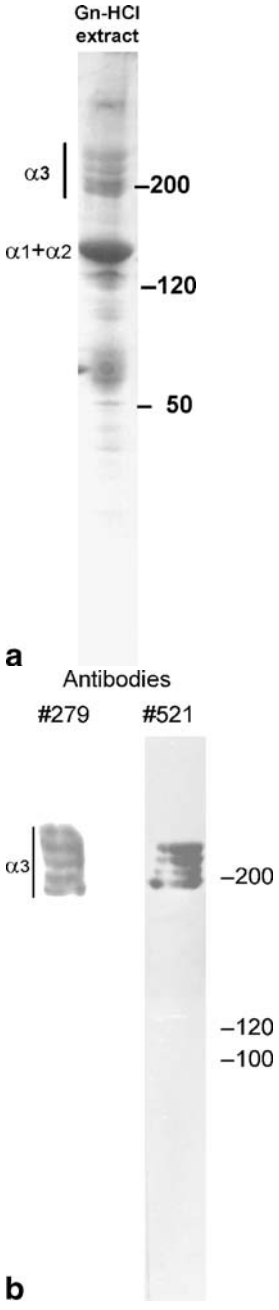

Fig. 1 Electrophoretic and immunochemical identification of type VI collagen in the Gn-HCl extract of the distal region of the deep flexor tendon (DFT) of the adult bovine. **a** SDS-PAGE under reducing conditions followed by staining with Coomassie blue; the Gn-HCl extract revealed a band of about 140 kDa, corresponding to the α1 and α2 chains, and a group of at least four bands with relative molecular mass of about 200 kDa, corresponding to the α3 chains. **b** Western blotting identification of type VI collagen α3 chains by using polyclonal antibodies nos. 279 and 521

reactive with the α3 chains (Fig. 1b). The presence of type VI collagen in the various tendons was assessed by SDS-PAGE of the two first Gn-HCl extracts. Reducing conditions led to the appearance of the bands corresponding to the α1(VI) and α2(VI) chains (Fig. 2). We were able to verify that type VI collagen was present in the DFT at the fetal, calf, and adult stages (in both the proximal and distal regions) and in the adult extensor digital tendon.

Purification of type VI collagen from the adult DFT

Type VI collagen was purified from the adult DFT by a combination of sieve and ion exchange chromatography. Figure 3 depicts two sequential steps in type VI purification. The elution pattern of the concentrated Gn-HCl extract after chromatography on a Sepharose CL4B column is shown in Fig. 3a. Type VI collagen appeared in many different fractions, as checked by SDS-PAGE under reducing conditions (Fig. 3b). The fractions containing type VI collagen were pooled, dialyzed against 7 M urea in 50 mM TRIS-HCl pH 8.3, and then subjected to anion-exchange chromatography on a DEAE-cellulose column. Lower pHs were tested but resulted in the aggregation of the components present in the pool. The bound material was eluted with three batches of increasing concentrations of NaCl. Type VI collagen was eluted from the column with low salt (0.2 M; Fig. 3c) whereas the large proteoglycans required higher salt concentrations. The purity of the type VI collagen preparation was checked by SDS-PAGE (Fig. 3d).

Rotary shadowing analysis

Rotary shadowing (Fig. 3e) indicated the predominance of type VI collagen. Although the SDS-PAGE revealed high purity, the rotary shadowing analysis indicated low contamination with pro-I or pro-III collagen. Rotary shadowing also revealed that type VI aggregates were short, up to 7 or 8 tetramers. These microfibrils were significantly shorter than those obtained from fetal tissues by sequential treatment with collagenase followed by gel sieve chromatography as used by others (Kielty et al. 1991), presumably because the denaturing conditions used here are known to disrupt the interactions that hold the tetramers together in microfibrils (Vogel and Meyers 1999).

Amount of type VI collagen in bovine tendons

The amount of type VI collagen in the various tendons and tendon regions with respect to animal age was determined by ELISA. The results were based in a standard curve prepared with purified type VI collagen. Linearity in the reaction product for type VI collagen was noted up to 2.5 μg/ml (Fig. 4a). As a control, we checked the reaction with purified type I collagen and aggrecan. Within the same concentration range, there was no reaction product, indicating no interference of these major components of the DFT in the experiment. The quantitative data obtained for the various samples is shown in Fig. 4b. A progressive accumulation of type VI collagen with age and a preferred accumulation in the distal region can be seen. The adult tensile region has a 5-fold accumulation of type VI compared with the corresponding region in the immature tendon. The distal fibrocartilaginous region accumulated a 6.5-fold greater amount of this collagen than the corresponding region of the immature tendon and contained 60% more type VI collagen than the proximal region at the adult stage. Both proximal and distal regions of the DFT contained significantly higher amounts of type VI collagen than did the adult extensor digital tendon.

Extractability

As we used a sequential extraction protocol, we could determine the extractability of type VI collagen from the various samples (Fig. 5). The first Gn-HCl extract brought about 96% of type VI collagen out of the proximal region of the adult DFT, ~87% from the adult distal region, and ~65% from the adult extensor digital. The remaining type VI collagen was extracted with the two subsequent rinses. The fetal and calf tendons were more resistant to the extraction of type VI collagen, with 40%–55% being extracted with the first Gn-HCl extraction (Fig. 5).

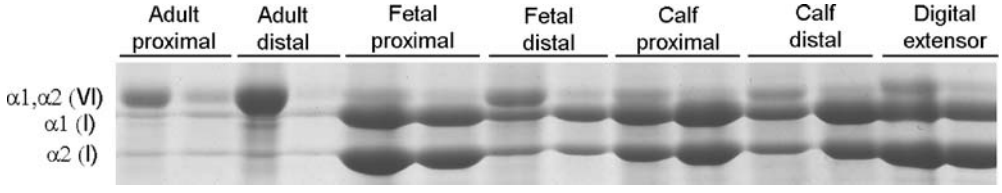

Fig. 2 Eletrophoretic pattern of the α1/α2 chains of type VI collagen in the various tendons and tendon regions after SDS-PAGE under reducing conditions and staining with Coomassie blue. Only the band corresponding to the α1/α2 chains of type VI collagen is shown, together with the bands for α1 and α2 of type I collagen. The two lanes (*left*, *right*) shown for each tendon/region correspond to the first and second Gn-HCl extracts, respectively (the third extract was omitted from this gel). Note that the extractability of type I collagen from the DFT is increased in older animals. This is not seen for the adult extensor digital tendon, which shows an intermediate amount of soluble type I collagen

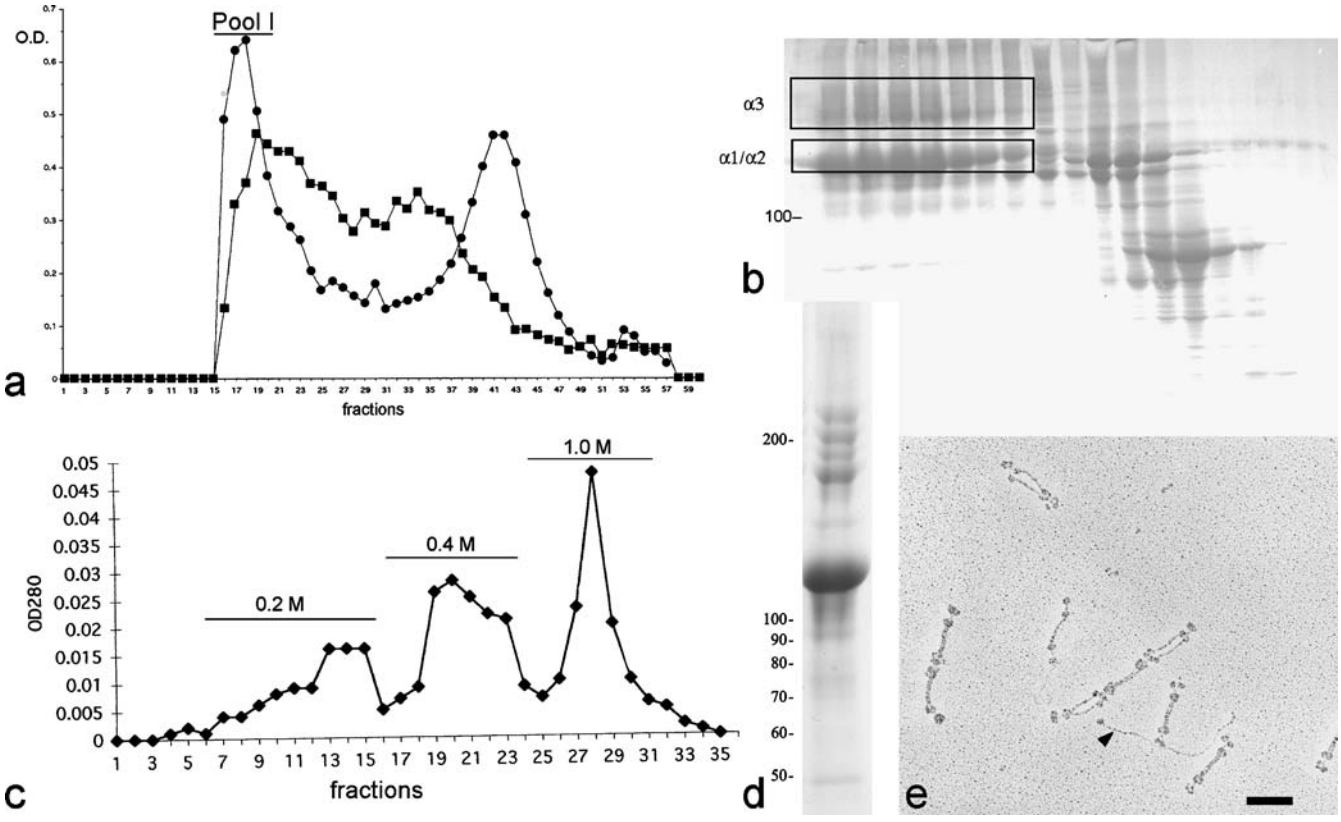

Fig. 3 Purification steps and characterization of type VI collagen from distal region of the adult DFT. **a** Distribution of proteins (optical density; OD 280, *circles*) and sulfated glycosaminoglycans (DMMB reaction, *squares*), in the fractions obtained by gel filtration chromatography of Gn-HCl extract of the distal region of adult DFT on a Sepharose CL4B column (2.5×100 cm). Fractions of the peak at the void volume (*Pool I*) were used in the subsequent purification. **b** SDS-PAGE analysis of fractions of the Sepharose CL4B chromatography, under reducing conditions. The migration positions of α1/α2 (Vl) and α3 (Vl) chains are indicated. **c** DEAE-cellulose chromatography of the material contained in the fractions indicated by Pool I in **a**. Type VI collagen was eluted from the column with 0.2 M NaCl. **d** SDS-PAGE under reducing conditions of the purified type VI collagen, after Coomassie blue staining. **e** Rotary shadowing analysis of the material eluted from the DEAE-column with 0.2 M NaCl, showing a predominance of type VI collagen aggregates, with the typical beaded string pattern with two or three tetramers. Longer (7–8) tetramers were present, but rare. Occasional pro-I or pro-III collagen (*arrowhead*) could be seen. *Bar* 100 nm

Distribution of type VI collagen in bovine DFT by immunohistochemistry

Type VI collagen was located by faint staining at the surface of collagen fibers in the tensile areas of the DFT (Fig. 6a,c,e). The epitenon and blood vessels showed stronger staining than the tendon mid-substance. Fetal, calf and adult tensile tendon showed this same staining pattern. The adult distal region showed more intense staining, with the reaction being observed at the surface of collagen fibers and also around the rounded fibrochondrocytes. Aligned fibrochondrocytes in lacunae and grouped cells (in niches) were both surrounded by a marked deposition of type VI collagen. At these latter structures, the staining for type VI was much stronger, extending over relatively wide areas (Fig. 6f).

Ultrastructure of the pericellular matrix and distribution of type VI collagen aggregates in adult DFT

Examination of the distal region of the DFT by transmission electron microscopy revealed large cells with dense cytoplasm rich in glycogen granules and lipid droplets. The cell outline was usually irregular, tending to a rounded format (Fig. 7a).

The pericellular matrix contained many different structural elements (Fig. 7b). Thin filaments connecting dense granules were seen and correlated with hyaluronan and aggregated proteoglycans. Thin microfibrils with a uniform diameter were seen in the middle of the hyaluronan/proteoglycan-rich region and probably consisted of type VI collagen, the banded appearance of which was often blurred in routine transmission electron microscopy preparations. Isolated and relatively thin collagen fibrils were scattered in the pericellular matrix. Deeper in the extracellular matrix, the collagen fibrils became thicker and more aggregated.

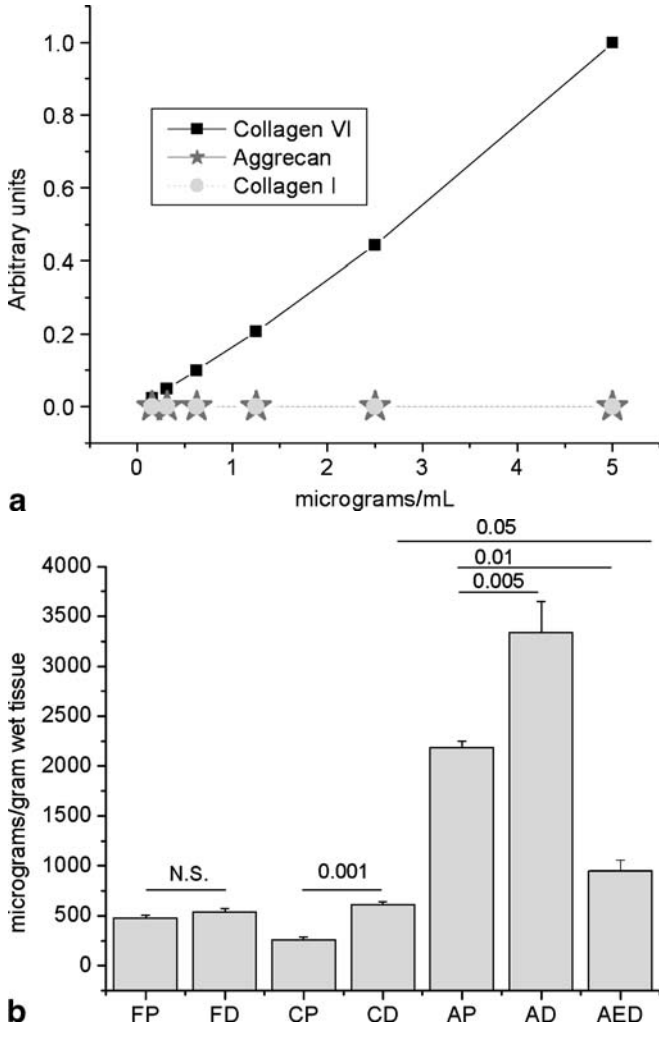

Fig. 4 ELISA for type VI collagen in bovine tendons. **a** Control experiment showing the linear response to increasing amount of type VI collagen up to 2.5 μg/ml. The reactions for type I collagen and aggrecan are also presented and showed no reaction with the antibody employed in the experiment, appearing superimposed at the baseline. **b** Amount of type VI collagen in the various tendon samples (mean ± standard error). The indicated P-values (*above* the *horizontal lines*) represent the level of significance of the differences between the mean content in each sample (*F* fetal, *C* calf, *A* adult, *P* proximal, *D* distal, *ED* extensor digital, *N.S.* non-significant)

ATP treatment resulted in the aggregation of type VI collagen and revealed the typical ladder-like structure with 110-nm periodicity. The aggregates formed a continuous layer around the rounded cells of the distal region (Fig. 7c). Detailed observation of its distribution revealed that the type VI collagen network extended from the pericellular layer into the spaces between adjacent collagen fibrils, firmly connecting both structures (Fig. 7d). We also noted that type VI collagen and fibrillin-based microfibrils lay side-by-side in regions in which elastin deposition occurred (Fig. 7e). The ATP treatment revealed the exclusion or scarcity of type VI collagen among densely packed collagen fibril in the proximal region of the DFT. However, immunocytochemistry at the electron microscopy level should be performed to ascertain type VI exclusion from the fibrous areas, as the ATP treatment may require a minimum concentration of type VI collagen to produce aggregates.

Discussion

In spite of the ubiquitous distribution of type VI collagen in tissues, its importance in muscles is more evident, since it has been firmly correlated with at least two types of muscular disorders: Betlhem muscular dystrophy and Ullrich muscular dystrophy. In both cases, mutations in the type VI collagen alpha chain genes result in either the absence or malformation of the microfibrils and, in each case, produce weak muscles, the diaphragm being the most commonly affected (Bonaldo et al. 1998; Camacho-Vanegas et al. 2001; Scacheri et al. 2002; Mercuri et al. 2002). The reason that muscles are the main tissues affected by type VI collagen deficiency is not clear. However, the lack of type VI collagen has been suggested to result in the weak interaction of the muscle fiber basal lamina with the adjacent connective tissue (Ishikawa et al. 2002). Although this hypothesis cannot be ruled out at present, more recently, a type VI collagen deficiency has been demonstrated to result in mitochondrial dysfunction and apoptosis (Irwin et al. 2003). In this case, seeding cells in a type VI substratum restores mitochondrial function and cell survival and treatment with cyclosporin A, an inhibitor of the mitochondrial permeability transition pore, also has a beneficial effect on cell survival (Irwin et al.

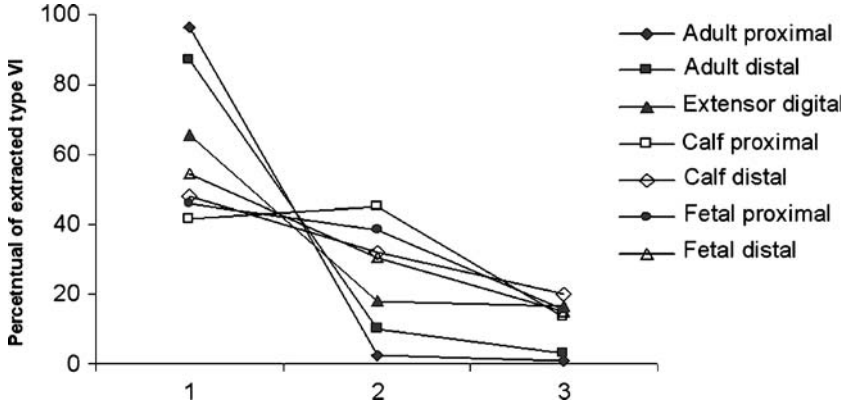

Fig. 5 Extractability of type VI collagen from the various tendon samples. The amount of type VI collagen in each of the three Gn-HCl extracts (*1-3*) was plotted as a fraction of the total amount of type VI collagen. Most of type VI collagen was readily extracted from the adult DFT tendons (~90% or more) with the first extraction round. The adult extensor digital tendon showed an intermediate extractability, whereas type VI was slowly extracted from the fetal and calf tendons

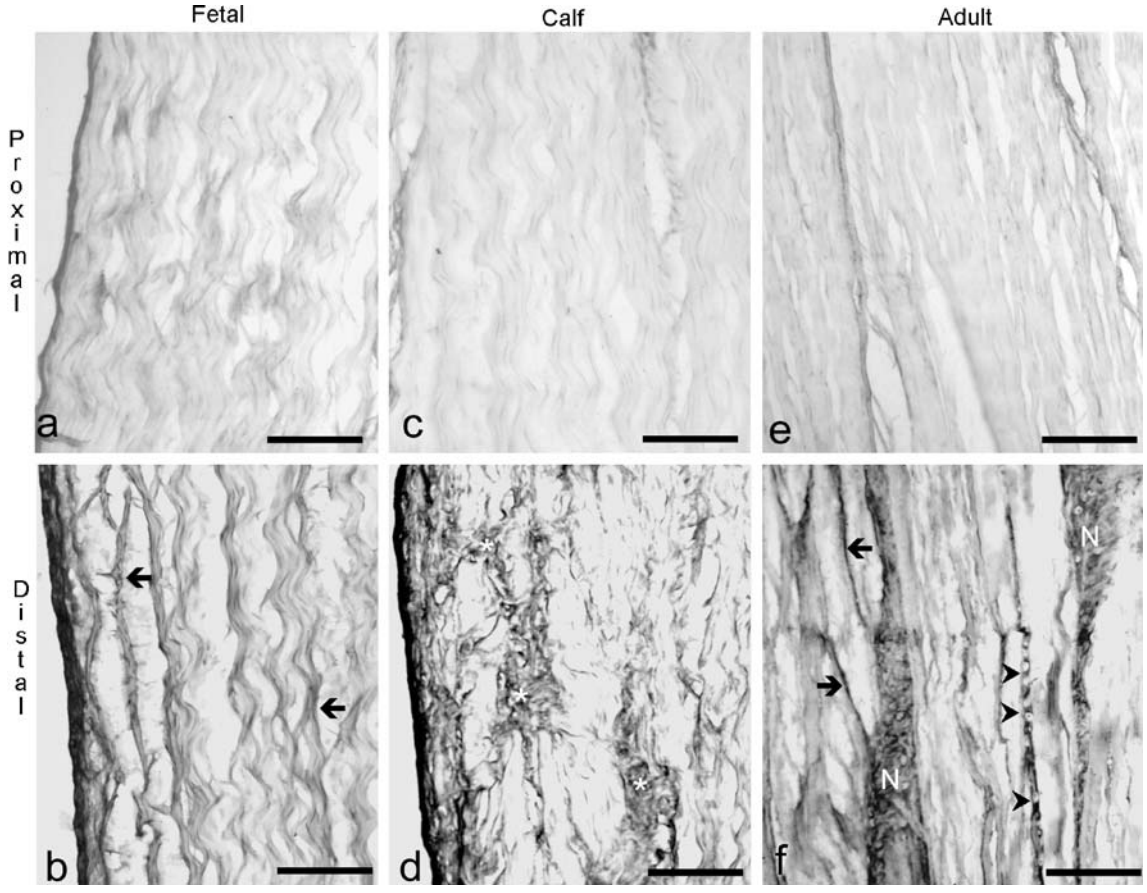

Fig. 6 Immunocytochemistry for type VI collagen in tendon samples. Frozen sections of fetal (**a, b**), calf (**c, d**), and adult (**e, f**) tendons were subjected to immunocytochemistry with a polyclonal antibody against type VI collagen. Type VI collagen was found in the proximal tensional regions (**a**, **c**, **e**) only at the surface of collagen fibers, at the external layers of the tendons, or around blood vessels. The immunocytochemical reaction was weak regardless of the age of the animal. The distal region showed a more intense accumulation of type VI. In the fetal tendon (**b**), type VI was more evident at the surface of the fibrils than in the proximal region (*arrows*). The calf tendon (**d**) demonstrated a non-uniform arrangement of the collagen fibers and an increased deposition of type VI collagen in the interfiber spaces and in some specific regions not occupied by collagen fibers (*white asterisks*). In the adult (**f**), type VI collagen accumulated around the rounded fibrochondrocytes either in lacunae (*arrowheads*) or in nests (*N*). It was also more evident in the interfiber spaces (*arrows*). *Bars* 50 μm

2003). The association between the extracellular microfibrils and the mitochondrion remains unknown. An examination of the signaling pathways downstream of the type VI collagen receptors, such as the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins (Timpl and Chu 1994; Loeser et al. 2000) and NG2 proteoglycan (Doane et al. 1998) would therefore be of interest.

Of note, type VI collagen may have tissue-specific functions, since Tanaka et al. (2003) have demonstrated a linkage between the COL6A1 gene on chromosome 21 and the ossification of the posterior ligament of the spine, a disease with high incidence in Japanese and other Asian populations. Furthermore, a possible association of type VI collagen overexpression with cardiac defects observed in Down syndrome patients has been suggested, given the location of the genes coding for both α_1(VI) and α_2(VI) chains in chromosome 21 (Barlow et al. 2001; Gittenberger-de Groot et al. 2003).

This work presents data on the presence, content, and distribution of type VI collagen in bovine tendons, together with protocols that result in the high purity of type VI collagen preparations and the accurate determination of its amount in tendons. Type VI collagen may constitute as much as 0.33% of the wet weight of the fibrocartilaginous region of the adult DFT. This is a considerable amount and is significantly higher than that in the proximal tensile region. The analysis of fetal and calf tendons has revealed a progressive accumulation of type VI collagen in tendons with age. The calf tendon shows a significantly higher amount of type VI collagen in the distal compressed region compared with that in the proximal tensile region. We have, however, observed a high variability in the content of type VI collagen in adult tendon, so that restriction of the age window for sampling is essential for consistency of quantitative data.

In the context of the present investigation, type VI collagen expression takes place after the fibrillogenesis stage in chicken tendons (Nurminskaya and Birk 1998) and, as mentioned before (Felisbino and Carvalho 1999), this is consistent with the interfibrillar and pericellular distribution of type VI collagen in tendons. Whether the temporal expression pattern described for the chicken

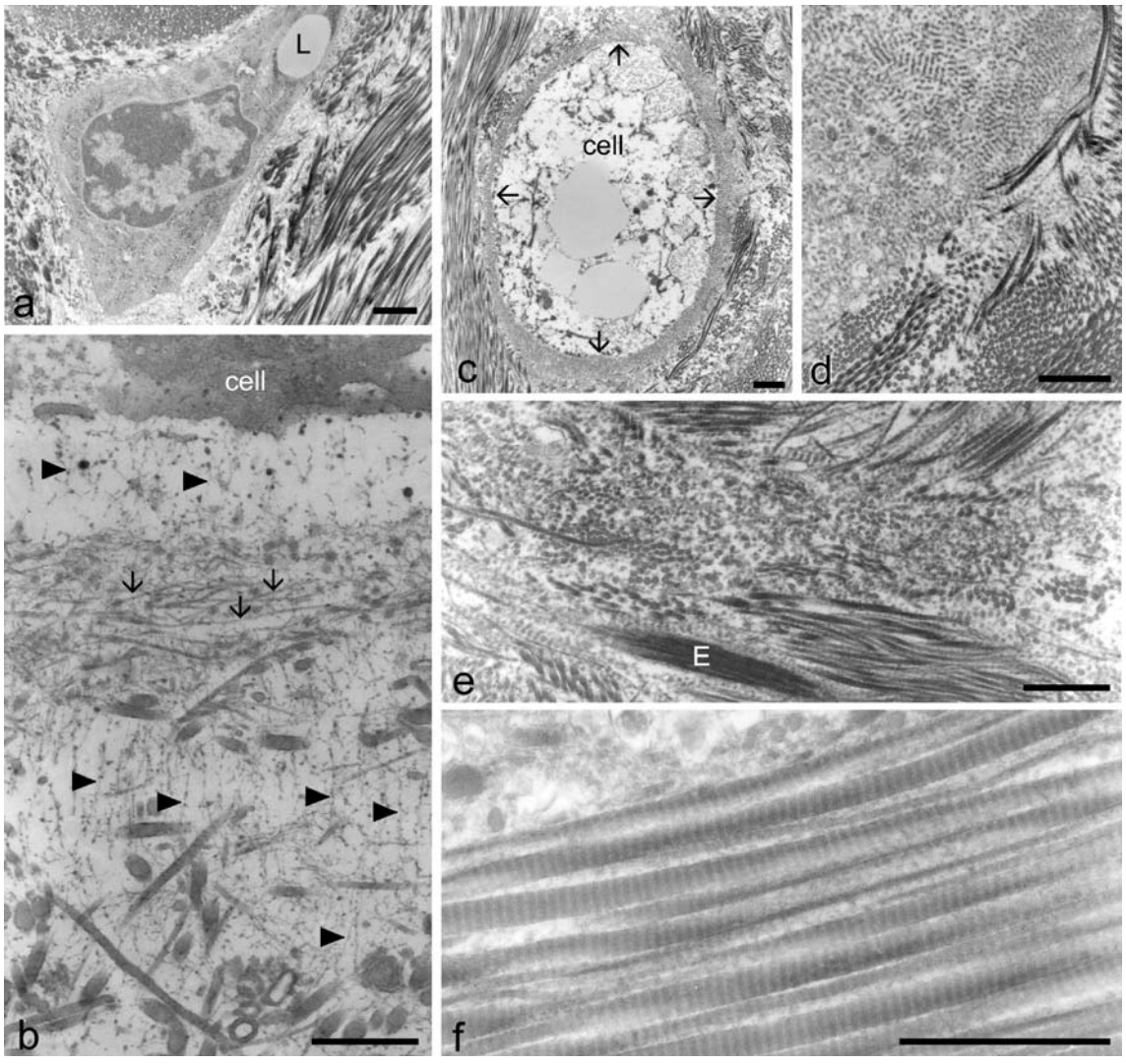

Fig. 7 Ultrastructural analysis of the tendon matrix and the identification of type VI collagen by ATP treatment in the adult DFT. **a** Micrograph of a cell in the distal region of the tendon. The cell has an irregular contour and rounded nucleus and exhibits both glycogen granules and a large lipid droplet (*L*). The organelles are mostly mitochondria and rough endoplasmic reticulum. The extracellular matrix presents collagen fibers and a relatively well-defined pericellular matrix. **b** Detail of the pericellular matrix in the distal region of the adult DFT. A net of thin filaments (*arrowheads*) connecting electron-dense granules of various sizes (possibly hyaluronan and collapsed proteoglycans) is observed throughout, radiating from the cell surface deep into the extracellular matrix. Close to the cell, a group of microfibrils (*black arrows*) can be oberved insterpersed with thin disperse collagen fibers. Deeper in the extracellular matrix, the collagen fibrils become thicker and aggregate into fibers. **c** En bloc ATP treatment results in aggregation of type VI collagen in the typical ladder-like structures with 110-nm periodicity. The aggregates form a continuous layer that lies around the fibrochondrocytes (*arrows*) and that delineates the pericellular matrix and extends into the adjacent spaces between collagen fibrils. **d** Detail of the accumulation of type VI collagen, with typical 110-nm banding, at the matrix between the pericellular space and the grouped collagen fibers. **e** Type VI collagen aggregates are also found away from the cells, among the collagen fibrils. They can also be observed close to fibrillin-based microfibrils, associated with elastin (*E*) deposition. **f** The tensional region is poor in type VI collagen, and aggregates of type VI collagen are rare. Type VI collagen is clearly excluded from the densely packed collagen fibrils groups in this region. *Bars* 1 μm

tendon applies to the bovine tendon and/or similar tendons in other species remains to be determined.

Surprisingly, the adult extensor tendons show a significantly reduced amount and low solubility of type VI collagen in comparison to the calf DFT. We have also observed a higher solubility of type I collagen from this tendon compared with the adult DFT. These differences may have important impacts in the biomechanics of the tendon and should be considered in explanations of the distinct properties of flexor and extensor tendons, in addition to other characteristics, such as water and sulfated glycosaminoglycan content (Batson et al. 2003). In addition, and more importantly, this difference in type VI collagen content and extractability in flexor vs. extensor tendons might reflect a special arrangement that would result in contractures of the distal joints; this appears as one of the main characteristic of type VI collagen disorders (Ishikawa et al. 2002).

The accumulation and distribution of type VI collagen around the fibrochondrocytes in the compressional areas of the adult DFT is similar to that observed for tendons under a similar biomechanical regimen in other species (Felisbino and Carvalho 1999), demonstrating the conservation of this pattern of extracellular matrix composition and organization. This pattern differs greatly from that seen in the tensional region, where type VI collagen is restricted to the external paratenon, to the matrix around blood vessels, and, to a certain extent, to the interfiber space. In the fibrocartilage, type VI might help to organize the pericellular matrix and may contribute to the ability of fibrochondrocytes to resist compressive forces in addition to tension. The ultrastructural analysis performed here has revealed that type VI collagen is present in a hyaluronan/proteoglycan-rich area in the distal region of the DFT. Moreover, the demonstration that type VI collagen has physical interactions with both type II collagen and aggrecan through matrillin−1 and biglycan or decorin in cartilage (Wiberg et al. 2003) reinforces the idea that it is important for the establishment of the pericellular matrix. In the case of the tendon fibrocartilages, type VI collagen might exert this function in addition to or in place of fibrillin−2, which has been suggested to organize the pericellular matrix in other tendons (Ritty et al. 2003).

In our opinion, the accumulation of type VI collagen is a good marker of fibrocartilage differentiation and may be produced as a response to compressive load. We further speculate that the function of type VI collagen in tendon may be to organize the pericellular matrix and that it may represent a survival factor for fibrochondrocytes. Furthermore, type VI collagen integrates the cellular environment with the adjacent fibrous mid-substance. An analysis of the tendons in type VI collagen-null mice or in muscular dystrophy patients might help to confirm this speculation and to reveal other possible functions of type VI collagen.

In conclusion, we have demonstrated that type VI collagen is present in bovine tendons in a tendon-specific and site-specific manner, that the fibrocartilage of the deep flexor digital tendon accumulates a large amount of type VI collagen during maturation and that it is concentrated around the cells. Furthermore, the extensor digital tendon has been shown to possess little type VI, and the type VI collagen in this tendon is less extractable than that of the corresponding flexors. The present results reinforce our previous suggestion that type VI collagen has an important function in organizing the pericellular environment for fibrochondrocytes (Felisbino and Carvalho 1999), thereby contributing to the differentiation and physiology of the pressure-bearing areas.

Acknowledgement The authors are grateful to Dr. Eva Engvall for donation of the antibodies. Some of this work was performed during H.F.C.′s stay in Albuquerque.

References

Barlow GM, Chen XN, Shi ZY, Lyons GE, Kurnit DM, Celle L, Spinner NB, Zackai E, Pettenati MJ, Van Riper AJ, Vekemans MJ, Mjaatvedt CH, Korenberg JR (2001) Down syndrome congenital heart disease: a narrowed region and a candidate gene. Genet Med 3:91–101

Batson EL, Paramour RJ, Smith TJ, Birch HL, Patterson-Kane JC, Goodship AE (2003) Are the material properties and matrix composition of equine flexor and extensor tendons determined by their functions? Equine Vet J 35:314–318

Bonaldo P, Braghetta P, Zanetti M, Piccolo S, Volpin D, Bressan GM (1998) Collagen VI deficiency induces early onset myopathy in the mouse: an animal model for Bethlem myopathy. Hum Mol Genet 7:2135–2140

Bruns RR, Press W, Engvall E, Timpl R, Gross J (1986) Type VI collagen in extracellular, 100-nm periodic filaments and fibrils: identification by immunoelectron microscopy. J Cell Biol 103:393–404

Camacho-Vanegas O, Bertini E, Zhang RZ, Petrini S, Minosse C, Sabatelli P, Giusti, B, Chu ML, Pepe G (2001) Ullrich scleroatonic muscular dystrophy is caused by recessive mutations in collagen type VI. Proc Natl Acad Sci USA 98:7516–7521

Carvalho HF, Vidal BC (1994a) Structure and histochemistry of a pressure-bearing tendon of the frog. Ann Anat 176:161–170

Carvalho HF, Vidal BC (1994b) The unique fibrillar arrangement of the bullfrog pressure-bearing tendon as an indicative of great functional deformability. Biol Cell 82:59–65

Covizi DZ, Felisbino SL, Gomes L, Pimentel ER, Carvalho HF (2001) Regional adaptations in three rat tendons. Tissue Cell 33:483–490

Doane KJ, Howell SJ, Birk DE (1998) Identification and functional characterization of two type VI collagen receptors, alpha 3 beta 1 integrin and NG2, during avian corneal stromal development. Invest Ophthalmol Vis Sci 39:263–275

Evanko SP, Vogel KG (1990) Ultrastructure and proteoglycan composition in the developing fibrocartilaginous region of bovine tendon. Matrix 10:420–436

Evanko SP, Vogel KG (1993) Proteoglycan synthesis in fetal tendon is differentially regulated by cyclic compression in vitro. Arch Biochem Biophys 307:153–164

Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphated glycosaminoglycans by use of dimethylmethylene blue. Biochim Biophys Acta 883:173–177

Felisbino SL, Carvalho HF (1999) Identification and distribution of type VI collagen in tendon fibrocartilages. J Submicrosc Cytol Pathol 31:187–195

Gillard GC, Reilly HC, Bell-Booth PG, Flint MH (1979) The influence of mechanical forces on the glycosaminoglycan content of the rabbit flexor digitorum profundus tendon. Connect Tissue Res 7:37–46

Gittenberger-de Groot AC, Bartram U, Oosthoek PW, Bartelings MM, Hogers B, Poelmann RE, Jongewaard IN, Klewer SE (2003) Collagen type VI expression during cardiac development and in human fetuses with trisomy 21. Anat Rec 275A:1109–1116

Irwin WA, Bergamin N, Sabatelli P, Reggiani C, Megighian A, Merlini L, Braghetta P, Columbaro M, Volpin D, Bressan GM, Bonaldo P (2003) Mitochondrial dysfunction and apoptosis in myophatic mice with collagen VI deficiency. Nat Genet 35:367–371

Ishikawa H, Sugie K, Murayama K, Ito M, Minami N, Nishino I, Nonaka I (2002) Ullrich disease: collagen VI deficiency: EM suggests a new basis for muscular weakness. Neurology 59:920–923

Kielty CM, Cummings C, Whittaker SP, Shuttleworth CA, Grant ME (1991) Isolation and ultrastructural analysis of microfibrillar structures from foetal bovine elastic tissues. Relative abundance and supramolecular architecture of type VI collagen assemblies and fibrillin. J Cell Sci 99:797–807

Koob TJ, Clark PE, Hernandez DJ, Thurmond FA, Vogel KG (1992) Compression loading in vitro regulates proteoglycan synthesis by tendon fibrocartilage. Arch Biochem Biophys 298:303–312

Loeser RF, Sadiev S, Tan L, Goldring MB (2000) Integrin expression by primary and immortalized human chondrocytes: evidence of a differential role for alpha1beta1 and alpha2beta1 integrins in mediating chondrocyte adhesion to types II and VI collagen. Osteoarthritis Cartilage 8:96–105

Mercuri E, Yuva Y, Brown SC, Brockington M, Kinali M, Jungbluth H, Feng L, Sewry CA, Muntoni F (2002) Collagen VI involvement in Ullrich syndrome: a clinical, genetic, and immunohistochemical study. Neurology 58:1354–1359

Merrilees MJ, Flint MH (1980) Ultrastructural study of tension and pressure zones in a rabbit flexor tendon. Am J Anat 157:87–106

Morris NP, Keene DR, Glanville RW, Bentz H, Burgeson RE (1986) The tissue form of type VII collagen is an antiparallel dimmer. J Biol Chem 261:5638–5644

Nurminskaya MV, Birk DE (1998) Differential expression of genes associated with collagen fibril growth in the chicken tendon: identification of structural and regulatory genes by subtractive hybridization. Arch Biochem Biophys 350:1–9

Okuda Y, Gorski JP, An KN, Amadio PC (1987) Related Articles, Links Biochemical, histological, and biomechanical analyses of canine tendon. J Orthop Res 5:60–68

Ritty TM, Roth R, Heuser JE (2003) Tendon cell array isolation reveals a previously unknown fibrillin-2-containing macromolecular assembly. Structure (Camb) 11:1179–1188

Robbins JR, Vogel KG (1994) Regional expression of mRNA for proteoglycans and collagen in tendon. Eur J Cell Biol 64:264–270

Rufai A, Benjamin M, Ralphs JR (1992) Development and ageing of phenotypically distinct fibrocartilages associated with the rat Achilles tendon. Anat Embryol (Berl) 186:611–618

Scacheri PC, Gillanders EM, Subramony SH, Vedanarayanan V, Crowe CA, Thakore N, Bingler M, Hoffman EP (2002) Novel mutations in collagen VI genes: expansion of the Bethlem myopathy phenotype. Neurology 58:593–602

Senga K, Kobayashi M, Hattori H, Yasue K, Mizutani H, Ueda M, Hoshino T (1995) Type VI collagen in mouse masseter tendon, from osseous attachment to myotendinous junction. Anat Rec 243:294–302

Tanaka T, Ikari K, Furushima K, Okada A, Tanaka H, Furukawa K-I, Yoshida K, Ikeda T, Ikegawa S, Hunt SC, Takeda J, Toh S, Harata S, Nakajima T, Inoue I (2003) Genomewide linkage and linkage disequilibrium analyses identify COL6A1, on chromosome 21, as the locus for ossification of the posterior longitudinal ligament of the spine. Am J Hum Genet 73:812–822

Timpl R, Chu M-L (1994) Microfibrillar collagen type VI. In: Yurchenco PD, Birk DE, Mecham RP (eds) Extracellular matrix assembly and structure. Academic Press, San Diego, pp 267–293

Vogel KG (1996) The effect of compressive loading on proteoglycan turnover in cultured fetal tendon. Connect Tissue Res 34:227–237

Vogel KG, Koob TJ (1989) Structural specialization in tendons under compression. Int Rev Cytol 115:267–293

Vogel KG, Meyers AB (1999) Proteins in the tensile region of adult bovine deep flexor tendon. Clin Orthop 367 (Suppl):S344–S355

Vogel KG, Peters JA (2005) Histochemistry defines a proteoglycan-rich layer in bovine flexor tendon subjected to bending. J Musculoskelet Neuronal Interact 5:64–69

Vogel KG, Keller EJ, Lenhoff RJ, Campbell K, Koob TJ (1986) Proteoglycan synthesis by fibroblast cultures initiated from regions of adult bovine tendon subjected to different mechanical forces. Eur J Cell Biol 41:102–112

Watanabe M, Kobayashi M, Fujita Y, Senga K, Mizutani H, Ueda M, Hoshino T (1997) Association of type VI collagen with D-periodic collagen fibrils in developing tail tendons of mice. Arch Histol Cytol 60:427–434

Wiberg C, Klatt AR, Wagener R, Paulsson M, Bateman JF, Heinegard D, Morgelin M (2003) Complexes of matrilin-1 and biglycan or decorin connect collagen VI microfibrils to both collagen II and aggrecan. J Biol Chem 278:37698–37704