Regular Paper

ISSN- 0102-9010

PHYSICOCHEMICAL AND STRUCTURAL ANALYSIS OF THREE REGIONS OF THE DEEP DIGITAL FLEXOR TENDON OF PIGS*

Vera Lúcia Corrêa Feitosa[1], Marcelo Augusto Marretto Esquisatto[2], Paulo Pinto Joazeiro[3], Laurecir Gomes[4], Sérgio Luis Felisbino[5] and Edson Rosa Pimentel[4]

[1]Department of Morphology, Federal University of Sergipe, Aracaju, SE, [2]Department of Histology, University of Araras, Araras, SP, [3]Department of Histology and Embryology and [4]Department of Cell Biology, State University of Campinas (UNICAMP), Campinas, SP, [5]Department of Morphology, Institute of Biosciences, Paulista State University (UNESP), Botucatu, SP, Brazil.

ABSTRACT

Few studies have discussed the relationship between the molecular organization and the physicochemical and biomechanical properties of pig tendons. In this work, we examined the extracellular matrix of the deep digital flexor tendon of pigs, which was subjected to tensional (proximal region) and compressive (distal and terminal regions) forces. The three regions of the tendon were used for swelling tests and their glycosaminoglycan content was determined. Longitudinal sections of the tendon were stained and observed using polarized light microscopy. The distal and terminal regions were swole more in water than the proximal region. After staining with toluidine blue the metachromasy was more intense in the distal and terminal regions, indicating an accumulation of proteoglycans in these regions. Analysis of the glycosaminoglycans by agarose gel electrophoresis showed that dermatan sulfate was present in all regions, whereas chondroitin sulfate occurred only in the regions of compression. The shape of the fibroblasts changed along the tendon: rounded cells occurred in regions under compression, while in the region under tension, elongated cells predominated. The organization and distribution of the collagen bundles were different for each region. Birefringence analysis revealed a more regular crimp pattern in the region under tension than in the regions under compressive forces. The elastic fibers also showed a different distribution in each region. These results indicate that the regional differences in the structure and composition of the deep digital flexor tendon of pigs are related to the biomechanical properties of the tendon.

Key words: Collagen, elastic fibers, pig tendon, pressure-bearing tendon, proteoglycan

INTRODUCTION

Tendons are dense, regularly arranged connective tissues consisting of spindle-shaped fibroblasts aligned between densely packed bundles of type I collagen fibers [5] and a very small amount of proteoglycans (PGs). Tendons transmit the tensional forces generated by muscle contraction to the bone on which they are inserted [29]. This function is dependent on the great tensile strength provided by collagen fibers [3]. Some tendons that pass under pulleys or a bone extremity also withstand compressive forces in addition to transmitting tension forces. In these tendons, the areas under the compressive forces develop a fibrocartilage [2,9] in which the elevated amount of PGs present is assumed to be the major factor in their ability to resist compression [29]. A distinct collagen fiber arrangement and the presence of elastic fibers both contribute to this resistance to compression [6].

Type VI collagen and other microfibrils are enriched in pressure-bearing regions in rabbit, dog, chicken, bullfrog and rat tendons [8,16]. These microfibrils are important in the microarchitecture and supramolecular organization, especially in the maintenance of the convoluted state of collagen fibers in the region of compression, and their crimp morphology in the region of tension [9,10,16].

Several studies have examined the correlation between the extracellular matrix (ECM) composition and the biomechanical properties of tendons in mammals such as rabbits [17,23], dogs [25,26], and cattle [13], as well as amphibians [7,9]. The deep digital flexor tendon (DDFT) of pigs is a

Correspondence to: Dr. Edson Rosa Pimentel
Departamento de Biologia Celular, Instituto de Biologia, CP 6109, Universidade Estadual de Campinas (UNICAMP), CEP 1308-3863 Campinas, SP, Brazil. Tel: (55) (19) 3788-6117, Fax: (55) (19) 3788-6111. E-mail: pimentel@unicamp.br

*This paper is dedicated to Professor Benedicto de Campos Vidal on the occasion of his 75th birthday.

wrap-around tendon that has three distinct regions, each experiencing different biomechanical forces. The proximal region is subject only to tensional forces whereas the distal region bifurcates into two branches towards the fingers and probably experiences compressive forces as it passes close to the metatarsophalangeal joint. The terminal region extends to the fingers and presumably withstands the compressive load at the site where the tendon attaches to the bone [4].

The purpose of this work was to correlate the PGs content, tissue swelling properties, structural features and macromolecular organization of the ECM with the biomechanical forces to which the DDFT of pigs is subjected.

MATERIAL AND METHODS

Animals

Eleven 45-day-old Large White male pigs, were obtained from the Center of Medicine and Experimental Surgery at UNICAMP. Eight pigs were used for the swelling tests and biochemical assays and three for morphological studies.

Biological material

The pigs were killed by overdose of 2.5% thiopental sodium (1 mL/kg body weight) and the hind limbs were dissected to obtain the DDFT. The tendon was divided into the proximal region, which experiences only tensional forces; the distal region, which forms two branches towards the fingers and is subject to some compressive forces as it passes close to the metatarsophalangeal joint, and the terminal region, which attaches to the distal phalange and experiences some compression (Fig. 1).

Swelling test

The different regions of the DDFT were cut in sequential cross-sections after which the specimens were equilibrated in 0.15 M NaCl, 0.05 M NaH_2PO_4, pH 7.0, for 1 h at room temperature, blotted and weighed. Each section was then soaked in a 1,000-fold excess volume of water for 60 min and weighed again. The sections were subsequently equilibrated in 3% acetic acid, pH 2.5 (1,000-fold excess volume) for 1 h, after which the wet weights were again determined [21].

Biochemical analysis

Papain digestion

Tendon fragments (0.05 g) were incubated with 500 µL of a papain solution (40 mg/g of tissue) in 0.03 M sodium citrate buffer containing 0.04 M EDTA and 0.08 M 2-mercaptoethanol, pH 3.5, for 24 h at 50°C, after which the resulting mixture was centrifuged in a Fischer microcentrifuge, at 8,000 rpm for 3 min. Glycosaminoglycans (GAGs) were precipitaded with two volumes of 95% ethanol for 24 h at 4°C. After centrifugation, the GAGs were washed sequentially in 80% ethanol and in acetone [24]. The GAGs liberated by papain digestion were dried at 37°C and identified by agarose gel electrophoresis in propilene diamine buffer (PDA) as described by Dietrich and Dietrich [12].

Quantification of sulfated GAGs

Sulfated GAGs were quantified using 100 µL of each digest (see above) per 2.5 mL of dimethyl methylene blue [14]. The amount of GAGs was determined using a standard curve prepared with whale chondroitin 4-sulfate (5, 10, 15, 20 and 25 µg/100 µL). The measurements were done in a Hewlett Packard 8452 A diode array spectrophotometer, at 526 nm.

Structural analysis

Histology and Histochemistry

Tendon fragments were fixed in 4% paraformaldehyde in Millonig's buffer, pH 7.4, for 24 h at room temperature. The material was then dehydrated in a graded ethanol series, clarified in xylene and embedded in Paraplast Plus. Serial longitudinal sections (6 µm thick) were stained with hematoxylin for 4 min and eosin for 1 min, and then differentiated in 70% ethanol for 1 min [1]. Some sections were stained with 0.025% toluidine blue in McIlvaine buffer at pH 4.0, for 20 min [22], to detect PGs. Collagen fibers were observed after staining with picrosirius solution (0.1% sirius-red F3B 200 in saturated picric acid solution), for 20 min [19], and counter-stained with Harris's hematoxylin for 10 min. To observe elastic fibers, the sections were oxidized in peracetic acid for 20 min, stained with Weigert's fuchsin-resorcin for 1 h and counterstained with picric acid for 5 min [18]. After dehydration, the slides were mounted in Entelan.

Statistical analysis

The results were expressed as the mean ± S.E.M., where appropriate, and were analyzed statistically by analysis of variance (ANOVA) and the Fischer distribution. A value of $P<0.05$ indicated significance.

RESULTS

Swelling properties

The swelling of the different regions of the DDFT is shown in Figure 2. When tendon fragments were transferred from PBS to distilled water (Fig. 2A), a marked increase in the wet weight was observed in the terminal region. The distal region showed an intermediate value whereas the proximal region shrank. The opposite behavior was noted when the fragments were soaked in acetic acid (Fig. 2B). In this case, the greatest increase in wet weight occurred in the proximal region.

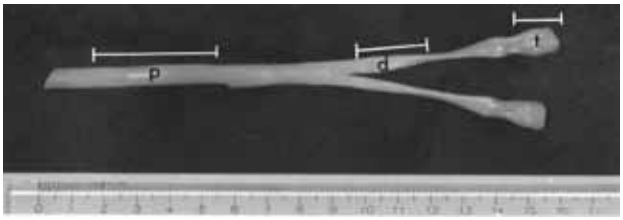

Figure 1. Dorsal view of the pig hind limb showing the location of the DDFT. The tendon was divided into proximal **(p)** and distal **(d)** regions that pass under the metatarsophalangeal joint, and terminal **(t)** region that inserts into the digits.

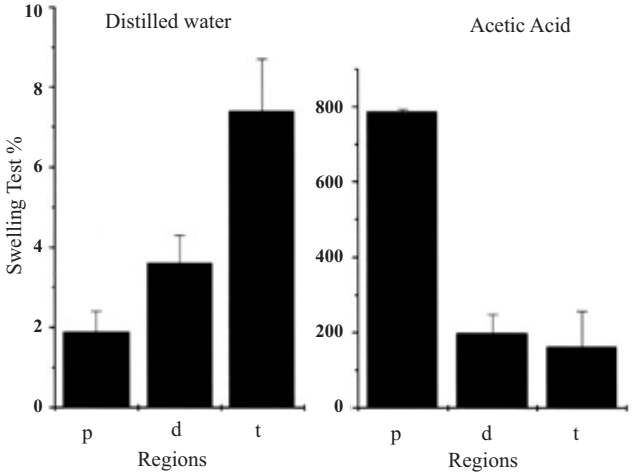

Figure 2. Swelling properties of the proximal **(p)**, distal **(d)** and terminal **(t)** regions of the DDFT, after soaking in distilled water for 1 h, and after incubation for 1 h in acetic acid (bottom). P<0.05 (ANOVA).

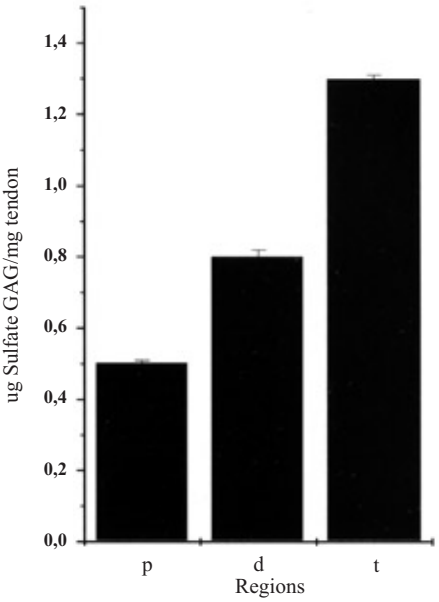

Figure 3. Content of sulfated GAGs in the proximal **(p)**, distal **(d)** and terminal **(t)** regions. P< 0.05 (ANOVA).

Analysis of sulfated GAGs

The total amount of sulfated GAG/g of wet tissue was determined after papain digestion of the three regions of the tendon, and was highest in the terminal region, followed by the distal region; the lowest amount was in the proximal region (Fig. 3).

The GAGs were identified by electrophoresis in agarose gels using a PDA buffer (Fig. 4). Dermatan sulfate (DS) occurred in all regions, whereas chondroitin sulfate (CS) was detected in the regions subjected to compression, particularly the terminal region. In the distal region only a faint band of CS was seen.

Structural aspects

Sections of the three regions stained with hematoxylin-eosin contained a large number of cells with different morphologies and distribution. In the proximal region (Fig. 5A), where only tensional forces are present, typical fibroblasts were arranged parallel to the collagen bundles. In the distal region (Fig. 5B), many rounded cells, similar to chondrocytes were seen between the elongated fibroblasts. The terminal region (Fig. 5C), which is subject to compressive and tensional forces, contained mainly chondrocyte-like cells especially in the area of contact with the bone.

When the sections were stained with toluidine blue at pH 4.0, basophilic material was detected in the three regions. In the proximal region (Fig. 5D), staining was seen in certain areas, preferentially around groups of fibroblasts; round cells were also present in some areas of this region (Fig. 5D). Metachromasy was restricted to some areas around groups of cells. In the distal region (Fig. 5E), metachromatic material was more widespread than in the proximal region. In the tensional region, the metachromasy was increased dramatically in the superficial portion, that is in contact with the bone

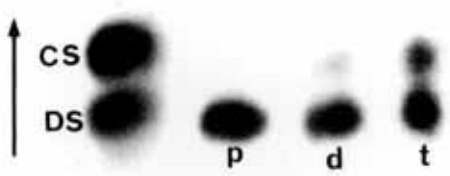

Figura 4. Agarose gel electrophoresis of GAGs obtained after papain digestion of the proximal **(p)**, distal **(d)** and terminal **(t)** regions of the DDFT. Dermatan sulfate **(DS)** was present in all regions of the DDFT, whereas chondroitin sulfate **(CS)** was present mainly in the distal and terminal regions. DS and CS standards are shown on the left. The arrow indicates the direction of migration.

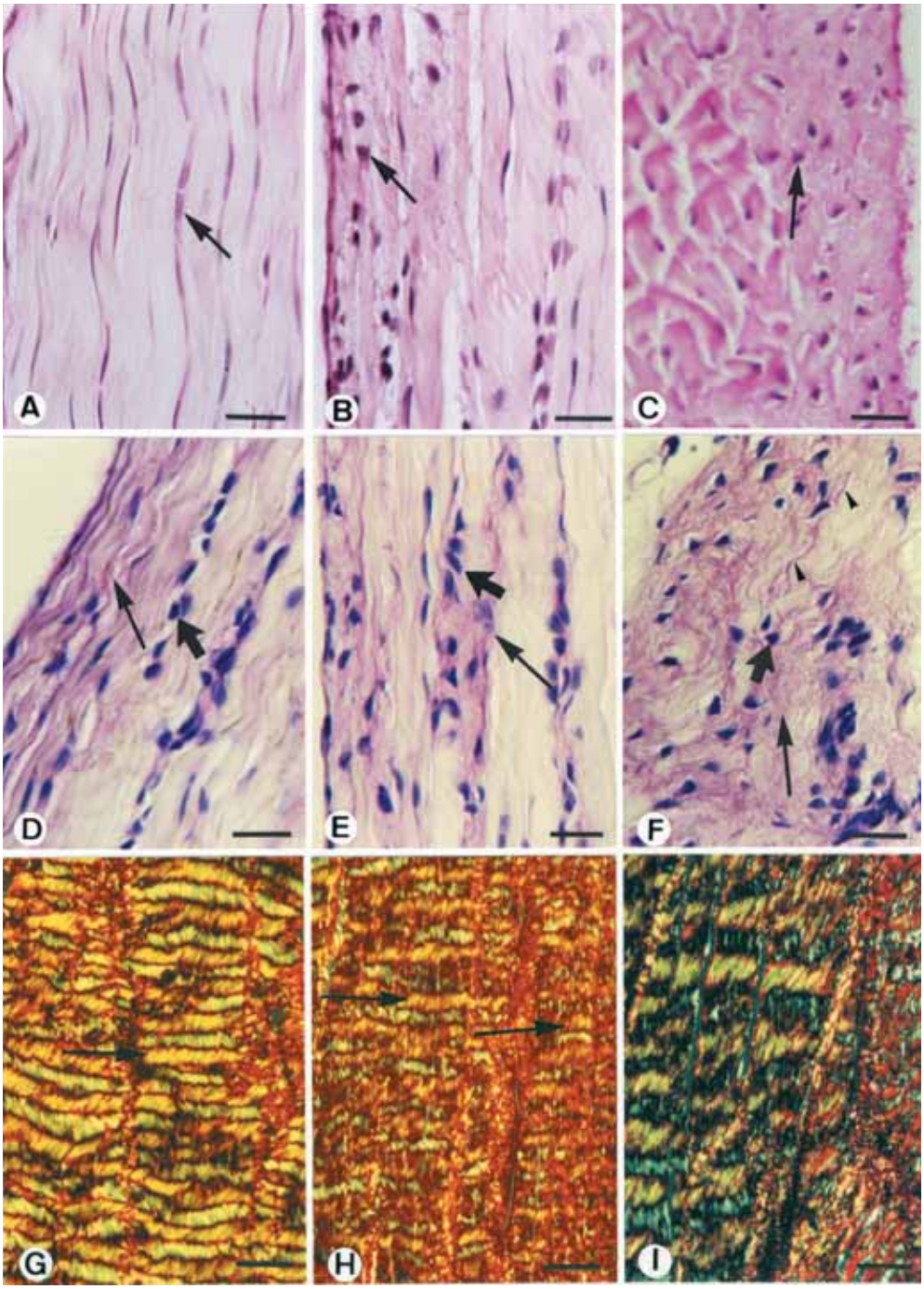

Figure 5. Longitudinal sections of the DDFT stained with HE **(A-C)**, toluidine blue **(D-F)** and picrosirius **(G-I)**. **A:** Proximal region showing fibroblasts **(→)** aligned with the collagen bundles. **B:** Aspect of the distal **(d)** region showing typical fibroblasts and rounded cells **(→)** similar to chondrocytes. **C:** Terminal region showing tendinocytes and rounded cells **(→)**. **D**: Proximal region showing weak **(→)** staining that was restricted to the areas with a higher concentration of these cells. Fibroblast nuclei (➔) are stained and demonstrate the organized distribution of cells in this region. **E:** Distal region showing rounded cells (➔) and a more intensely stained ECM **(→)**, especially in areas with a higher number of cells. **F:** Terminal region showing rounded cells (➔) and metachromatic ECM **(→)**. Note the presence of fibril bundles (➤) distributed in several directions. **G:** Proximal region showing intense birefringence. The undulated collagen fibers are organized side by side along the main axis of the tendon. Crimp **(→)** was more regular than in the distal and terminal regions. **H:** Distal region that bears some of the force of compression. Note that the collagen fibers were undulated and had a different crimp pattern **(→)** from that of the proximal region. **I:** Region of compression region showing the arrangement of the collagen fibers, which are less organized. The birefringence colors (yellow, green and red) indicate different levels of compactation of the collagen bundles. Bars = 50 μm (A-F) and 200 μm (G-I).

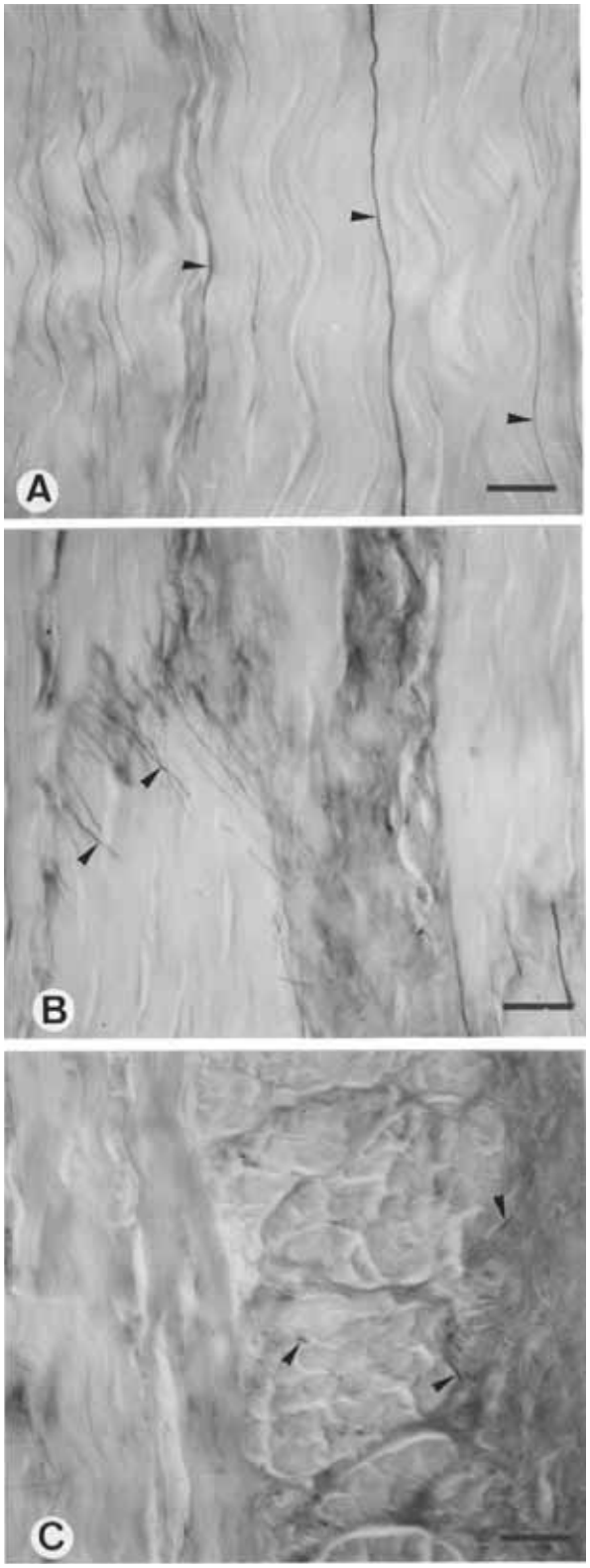

Figure 6. Distribution of elastic fibers in the proximal, distal and terminal regions of the DDFT. **A**: In the proximal region, the elastic fibers **(arrowheads)** follow the undulated morphology of the collagen bundles. **B** and **C**: Distal and terminal regions, respectively, where the elastic fibers **(arrowheads)** are distributed in several directions. Bars = 50 μm.

(Fig. 5F). The wavy aspect of the collagen bundles was clearly observed, and the ECM was not as organized as in the proximal and distal regions (Fig. 5D,E). Round cells were more frequent below the articulating surface (Fig. 5F).

The differences in the organization of the ECM of the three regions were seen better under polarized light in sections stained with picrosirius. A strong birefringence was observed in the three regions. The crimp structure was detected in all regions, but its pattern varied among them. In the proximal region, the collagen bundles were arranged in a highly ordered manner, and produced a crimp morphology of constant size and periodicity (Fig. 5G). Different arrangements were observed in the distal (Fig. 5H) and terminal (Fig. 5I) regions. In the distal region, there was no periodicity in the crimp structure, even though the fibrillar components were strongly stained by picrosirius and hematoxylin. In the terminal region, there was a predominant greenish interference color and a non-uniform crimp morphology.

The elastic fibers found along the tendon, showed different distribution patterns in each region. In the region of tension (Fig. 6A), they were arranged in the same direction as the collagen bundles. In the distal region (Fig. 6B), the elastic fibers were parallel and oblique to the long axis of the tendon. A similar distribution was seen in the terminal region (Fig. 6C).

DISCUSSION

Wrap-around tendons experience compressive and frictional forces, in addition to the tensional forces originating from the muscle [29]. To withstand the compressive forces, these tendons have developed a fibrocartilage that has structural and functional properties intermediate to those of dense fibrous connective tissue and hyaline cartilage [2].

The pig DDFT is a wrap-around tendon that has a proximal fibrous region that is subjected only to tensional forces, a distal region, that bifurcates and articulates with the metatarsophalangeal joint, and a terminal region, that inserts into the fingers. The latter two regions experience compressive forces.

Physicochemical and biochemical procedures as well as structural analyses were used to study each of these regions. The greatest swelling in water occurred in the distal and terminal regions, and was indicative of the presence of PGs; this was confirmed by the quantification of GAGs after papain digestion.

In contrast, when the tendon was soaked in acetic acid, the greatest swelling occurred for the proximal region and was correlated with the lowest content of PGs, as shown by the quantification of sulfated GAGs. Similar results have been reported for the flexor tendon of cattle [21]. The increased content of PGs in the distal and terminal regions compared to the proximal region indicates the importance of PGs in providing osmotic resistance to compressive loads [21].

Agarose gel electrophoresis showed that DS was present in all regions of the DDFT, while CS was seen only in the terminal region. Our results confirm previous data showing the presence of a small proteoglycan containing DS in a highly collagenous tissue that is under constant tensional forces, and the presence of CS in regions under compression in rabbits [17,23], cattle [30] and amphibians [7,9].

Morphological analysis showed the presence of groups of round cells and a strong metachromatic basophilia in the distal and terminal compressive regions, as opposed to the elongated fibroblasts and little metachromasy in the region of tension. The presence of round cells in some areas of the latter region was indicative of the immaturity of this tendon since we used 45-day-old pigs. The regions of tension in tendons of newborn rats, contain round cells [27], that subsequently change to elongated cells, following the unidiretional arrangement of the collagen bundles. In 45-day-old pigs, some cells remained unchanged. The metachromasy seen in the two pressure-bearing areas was consistent with the higher content of GAG detected by the DMMB procedure as compared to the proximal tension-bearing region.

Polarized light microscopy showed that the organization of the collagen fibers was not uniform in the three regions, indicating that they are adapted to different biomechanical forces. The crimp morphology in the proximal region reflected the supramolecular helical arrangement of the collagen bundles, the importance and implications of which have been extensively analyzed by Vidal [28]. The distal and terminal regions contained collagen fibers arranged in a three dimensional network, in various directions and showed a less uniform crimp morphology. A different arrangement was observed in the superficial digital flexor tendon (SDFT) of pigs [15], which also experiences compressive forces. The differences between the compressive areas of the SDFT and the DDFT probably reflect the peculiar biomechanical properties of these two tendons. The compressive area of the former tendon wraps around the tibio-tarsal joint, whereas the terminal region of the DDFT is a fibrocartilaginous structure that enables this region to dissipate stress at the tendon-phalange interface. The molecular signaling mechanism that transmits the alterations in the extracellular environment to the cell is still incompletely understood, although some mechanosensory molecules at the cell surface have been identified [11,20].

Variation in the elastic fiber content and distribution is an additional factor that distinguishes the tensional and compressive regions of the pig tendon. The elastic fibers were aligned to the collagen fibers in the proximal region and showed no preferred orientation in the compressive distal and terminal regions, where they can accomodate deformation in several directions. A similar distribution of elastic fibers in different tendon areas has also been observed in wrap-around tendons of the bullfrog [9].

In conclusion, our results show that the DDFT of pigs contains compositional and structural variations among the different regions, and that these variations are directly related to the capacity to support compression and to transmit tension forces.

ACKNOWLEDGMENTS

The authors thank the Center for Medicine and Experimental Surgery of the Faculty of Medical Sciences, UNICAMP, for supplying the pigs. The authors thank H. F. Carvalho for the use of his Carl Zeiss Axioskop Microscope and for careful reading of this manuscript. V. L. C. Feitosa was supported by a CAPES-PICDT fellowship.

REFERENCES

1. Behmer AO, Tolosa EMC, Freitas Neto AG (1976) *Manual de Técnicas para Microscopia Normal e Patológica*. 1st ed. Edusp: São Paulo.
2. Benjamin M, Evans EJ (1990) Fibrocartilage. *J. Anat.* **171,** 1-15.
3. Benjamin M, Ralphs JR (1998) Fibrocartilage in tendons and ligaments – an adaptation to compressive load. *J. Anat.* **193,** 481-494.
4. Benjamin M, Ralphs JR (2000) The cell and developmental biology of tendons and ligaments. *Int. Rev. Cytol*. **196,** 85-130.
5. Bloom W, Fawcett DW (1994) *A Textbook of Histology*. 12th ed. Chapman & Hall: London.
6. Carvalho HF (1995) Understanding the biomechanics of tendon fibrocartilages. *J. Theor. Biol*. **172**, 293-297.

7. Carvalho HF, Felisbino SL (1999) The development of the pressure-bearing tendon of the bullfrog, *Rana catesbeiana*. *Anat. Embryol*. **200**, 55-64.
8. Carvalho HF, Neto JL Taboga SR (1994) Microfibrils: neglected components of pressure-bearing tendons. *Ann. Anat.* **176**, 155-159.
9. Carvalho HF, Vidal BC (1994) Structure and histochemistry of a pressure-bearing tendon of the frog. *Ann. Anat*. **176**, 161-170.
10. Carvalho HF, Vidal BC (1995) The elastic system of a pressure-bearing tendon of the bullfrog *Rana catesbeiana*. *Ann. Anat*. **177**, 397-404.
11. Chiquet M, Renedo AS, Huber F, Flück M (2003) How do fibroblasts translate mechanical signals into changes in extracellular matrix production? *Matrix Biol.* **22**, 73-80.
12. Dietrich CP, Dietrich SMC (1976) Electrophoretic behaviour of acidic mucopolysaccharides in diamine buffers. *Anal. Biochem.* **70**, 645-647.
13. Evanko SP, Vogel KG (1990) Ultrastructure and proteoglycan composition in the developing fibrocartilaginous region of bovine tendon. *Matrix* **10**, 420-436.
14. Farndale RW, Buttle DJ, Barrett AJ (1986) Improved quantitation and discrimination of sulphate glycosaminoglycans by use of dimethylmethylene blue. *Biochim. Biophys. Acta* **883**, 173-177.
15. Feitosa VL, Vidal BC, Pimentel ER (2002) Optical anisotropy of a pig tendon under compression. *J. Anat*. **200**, 105-111.
16. Felisbino SL, Carvalho HF (1999) Identification and distribution of type VI collagen in tendon fibrocartilages. *J. Submicrosc. Cytol. Pathol*. **31**, 187-195.
17. Gillard GC, Reilly HC, Bell-Booth PG, Flint M.H (1979) The influence of mechanical forces on the glycosaminoglycan content of the rabbit flexor digitorum profundus tendon. *Connect. Tissue Res*. **7**, 37-46.
18. Goldfischer S, Coltoff-Schiller B, Schwartz E, Blumenfeld OO (1983) Ultrastructure and staining properties of aortic microfibrils (oxytalan). *J. Histochem. Cytochem*. **31**, 382-390.
19. Junqueira LCU, Bignolas G, Brentani R (1979) Picrosirius staining plus polarization microscopy, specific methods of collagen detection in tissue section. *Histochem. J*. **11**, 447-455.
20. Kjaer M (2004) Role of extracellular matrix in adaptation of tendon and skeletal muscle to mechanical loading. *Physiol. Rev.* **84**, 649-698.
21. Koob TJ, Vogel KB (1987) Site-related variations in glycosaminoglycan content and swelling properties of bovine flexor tendon. *J. Orthop. Res*. **5**, 414-424.
22. Mello MLS, Vidal BC (1980) *Práticas de Biologia Celular*. Edgard Blücher: São Paulo.
23. Merrilees MJ, Flint MH (1980) Ultrastructural study of tension and pressure zones in a rabbit flexor tendon. *Am. J. Anat.* **157**, 87-106.
24. Michelacci YM, Horton DSPQ (1989) Proteoglycans from the cartilage of young hammerhead shark *Sphyrna lewini*. *Comp. Biochem. Physiol*. **92B**, 651-658.
25. Okuda Y, Gorski KN, Amadio PC (1987a) Biochemical, histological, and biomechanical analysis of canine tendon. *J. Orthop. Res*. **5**, 60-68.
26. Okuda Y, Gorski KN, Amadio PC (1987b) Effect of postnatal age on the ultrastructure of six anatomical areas of canine flexor digitorum profundus tendon. *J. Orthop. Res*. **5**, 231-241.
27. Vidal BC, Mello MLS (1984) Proteoglycan arrangement in tendon collagen bundles. *Cell. Mol. Biol*. **30**, 195-204.
28. Vidal BC (2003) Image analysis of tendon helical superstructure using interference and polarized light microscopy. *Micron* **34**, 423-432.
29. Vogel KG, Koob TJ (1989) Structural specialization in tendons under compression. *Int. Rev. Cytol*. **115**, 267-293.
30. Vogel KG, Sandy JD, Pogány G, Robbins JR (1994) Aggrecan in bovine tendon. *Matrix Biol*. **14**, 171-179.

Received: November 24, 2004
Accepted: January 26, 2005