FULL PAPER
Anatomy

Morphometric analysis of growing tenocytes in the superficial digital flexor tendon of piglets

Naoki TAKAHASHI[1), Prasarn TANGKAWATTANA[1,2)*, Yoshiki OOTOMO[1), Takuya HIROSE[1), Jun MINAGUCHI[1), Hiromi UEDA[1), Michi YAMADA[3) and Kazushige TAKEHANA[1)

[1)Laboratory of Microanatomy, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkiado 069-8501, Japan
[2)Department of Veterinary Anatomy, Faculty of Veterinary Medicine, Khon Kaen University, Khon Kaen 40002, Thailand
[3)College of Agriculture, Department of Food and Environment Science, Rakuno Gakuen University, Ebetsu, Hokkaido 069-8501, Japan

ABSTRACT. The fine structures of different tendons in various animals at different ages have been studied extensively to reveal their arrangement and growth patterns. However, knowledge of the microstructures of the growing tenocytes in the tendons of piglets is still lacking. Thus, we performed the first morphometric analysis to describe the characteristics of tenocytes in the metacarpal superficial digital flexor tendon of 0-, 10- and 20-day-old piglets. In the present study, hydrochloric acid/collagenase digestion was applied to remove the interstitial connective tissue to obtain clear visualization of intact tenocytes and their cytoplasmic processes (Cp). Then, the morphometry of the tenocytes was investigated by optical and electron microscopy. The mean ± SE values of the fascicle area, number of tenocytes/fascicle, cell density, number of Cp/tenocyte, length of Cp, and thickness of Cp were compared among the three age groups. Significant differences (judged at $P<0.05$) were found in almost all morphometric aspects among the age groups, except for the number of Cp/cell ($P=0.545$) and thickness of the Cp ($P=0.105$). A decrease of cell density corresponded with an increase in the length of the Cp, which were extended to connect either with the Cp of the other tenocytes or the surrounding endotendineum. Moreover, an increase of the fascicle area reflected the increase in tendon diameter. The revealed morphometric characteristics are thus the outcome of tendon growth.

KEY WORDS: morphometry, piglets, superficial digital flexor tendon, tenocyte

The tendon is a densely and regularly arranged group of collagen fibers mediating the attachment of the skeletal muscle to the bone. As such, the tendon plays significant roles in transmitting tension from the muscle to the bone, producing force in addition to that being produced by muscular contraction [20], regulating the articular position, and protecting the muscle fibers from damage [15]. Approximately 90–95% of the cell population in the tendon are tenocytes, which are tendon cells that localize along the longitudinal orientation of the collagen fibers [9]. Tenocytes are known to be responsible for producing collagen fibers, proteoglycans, degradation enzymes, and cytokines to maintain the dynamic equilibrium of both the fibrous and non-fibrous components of the extracellular matrix (ECM). These components are usually similar to other connective tissues, except that they contain more abundant collagen fibers that follow a course along a hierarchical pattern toward their bony destinations. The collagen fibers in each tendon generally assemble into fascicles, each of which is surrounded by the endotendineum, which is the intratendinous trabeculae of the peritendineum. Such arrangement is a fundamental property of the tendon to confer it with flexibility and resistance to high tension [13].

The extent of productivity of the collagenous population seems to be related to the age of animals. In murine species, an increase in the number of collagen fibrils was detected only in the fetal stage, but the length and diameter of these fibrils increased postnatally [8]. An increase in the diameter of collagen fibrils and the ratio of collagen fibrils per unit area in the tendon of porcine species with age have also been reported. In porcine, collagen fibrils start weaving to form collagen fibers at birth, but the parallel orientation of these fibers is established subsequently. The thickness of the individual collagen fibril in 6-month-old

pigs was reported to be twice (52–102 μm) that of newborn piglets. This increase of collagenous components would result in a corresponding increase of the total length and cross-sectional area of the tendon. Thus, we speculated that age might be one of the major factors contributing to the observed differences in tendinous generation. Since the growth of the tendon is surely related to the activity of tenocytes, investigating the morphometry of the tenocytic population in the tendon of growing animals should yield a better understanding of the growth pattern of tenocytes.

It is well established that Cp of tenocytes are extended in all directions to connect with either the collagen bundle of the endotendineum or Cp of the vicinal tenocytes [2, 21]. Variation and different amounts of collagenous components would affect the morphometry of the tenocytes, including the length and thickness of their Cp, in the tendons of each animal species [7]. The tenocytes in the tendons of the adult rat tail were found to vary in size, with a range of 4–7 μm in width and 15–25 μm in length; moreover, the Cp were approximately 3 μm or less in length [22]. However, it is difficult to conduct microscopic observations of tenocytes and their processes in an intact tendon since the interstitial connective tissues can interfere with the visual information. Therefore, ample elimination of these fibrous components should alleviate the challenges associated with the morphometric investigation of tenocytes. A connective tissue digestion technique using hydrochloric acid (HCl) and collagenase has been verified to effectively remove these extracellular components without damaging the cellular components of any chemically fixed tissues [15]. These two chemicals were first used for the investigation of collagen fibers and the basement membrane in biopsied samples. The technique was then modified for removing extracellular substances to yield better information of the tenocytes [6]. Thus far, one study has applied this technique to investigating the Achilles tendon of the rat [15]. Therefore, we sought to conduct the first study using a similar technique to facilitate the morphometric analysis of tenocytes and their Cp in the superficial digital flexor tendon (SDFT) in the forelimb of piglets aged 0, 10 and 20 days.

MATERIALS AND METHODS

Animals

Fifteen (five 0-day-old, five 10-day-old and five 20-day-old) crossbred (Land Race × Large Yorkshire × Duroc) piglets of the Rakuno Gakuen University farms were used in this study. Animal experiments strictly conformed to the Laboratory Animal Guidelines of the Experimental Animal Committee of Rakuno Gakuen University (approval number: VH14C4).

Tendon collection

Anesthesia was performed by the intraperitoneal administration of 20 mg/kg pentobarbital (Somnopentyl®, Kyoritsu Pharmaceutical, Tokyo, Japan). The animals were euthanized by exsanguination and then the SDFT coursing behind the metacarpus was collected.

Removal of extracellular connective tissue

Five 5-mm-thick sections from each tendon were transversely resected with a sharp razor blade. The blade was wiped with absolute ethanol prior to resecting so as to remove any moisture, grease, dust, and rust that might contaminate the tendons. The resected samples of each tendon were placed in separate test tubes and fixed with 3.0% glutaraldehyde with 0.1 M phosphate-buffered saline (PBS), pH 7.4, overnight at room temperature. The sections were then washed three times consecutively for 10 min each in 0.1 M PBS with mild shaking. The PBS was then replaced by 6 N HCl and digestion was conducted for 15 min in a 60°C water bath with periodical shaking. Thereafter, three more consecutive 10-min washings with PBS were carried out in the water bath at 60°C for the first two washings and then at 30°C for the third wash. The PBS was then replaced by a collagenase solution prepared by dissolving 1 mg/ml collagenase (Brightase-C, Nippi Co., Tokyo, Japan) in a buffer solution containing 50 mM Tris-HCl, 200 mM NaCl and 5 mM CaCl$_2$. The digestion was performed in the 30°C water bath for 12 hr, followed by three consecutive 10-min washes with PBS in the 30°C water bath. These samples were used for further investigations by optical microscopy and electron microscopy.

Scanning electron microscopy

The digested samples were post-fixed for 1 hr with 1.0% osmium tetroxide, followed by three consecutive 10-min PBS washes. Thereafter, a series of treatments and washes were performed to obtain conductive dyeing: 1% tannic acid for 30 min, three consecutive 10-min PBS washes, 1% osmium tetroxide for 1 hr, and three consecutive 10-min PBS washes. Dehydration with an ethanol series was carried out for 30 min at each concentration, followed by three consecutive 30-min dehydrations in 100% ethanol. The samples were further treated with a mixture of 100% ethanol and t-butyl alcohol (1:1) for 30 min, and then with only t-butyl alcohol for 30 min three times. After freezing, the samples were freeze-dried in a freeze dryer (JFD-300; JEOL Ltd., Tokyo, Japan). The samples were ion-coated with platinum using a magnetron sputtering apparatus (JUC-5000; JEOL Ltd.). A scanning electron microscope (JSM-5200, JEOL Ltd.) was used at an acceleration voltage of 20 kV to confirm the successful removal of the interstitial connective tissue and number of Cp.

Optical and transmission electron microscopy

The digested samples were post-fixed with a 1.0% osmium tetroxide solution, dehydrated with an ethanol series, and embedded in Quetol 812 (Nisshin EM, Tokyo, Japan). An ultramicrotome (Reichert Supernova, Leica Microsystem, Tokyo, Japan) was used for preparing semi-thin (1.0–1.5 μm) and ultra-thin (80 nm) sections. The semi-thin sections were mounted on glass slides, stained

with toluidine blue, and observed with an optical microscope for determination of the number of tenocytes/fascicle and the fascicle area. The ultra-thin sections were mounted on a 200-mesh copper grid, dried, and counter-stained with 1% uranyl acetate for 1 min and then with 2% lead citrate for 5 min. A transmission electron microscope (JEM-1220; JEOL Ltd.) was applied at an accelerating voltage of 80 kV to investigate the length, thickness, and adhering termini of the Cp.

Morphometric analysis of tenocytes

Image J analysis software (version 1.48v, National Institutes of Health, Bethesda, MD, U.S.A.) was used for the morphometric analysis of tenocytes observed by optical and electron microscopy. On the optical micrographs, five fascicles in each semi-thin section were randomly selected for measuring the area of the fascicles (μm^2) and to count the number of tenocytes per fascicle. Then, the cell density (number of tenocyte/10^4 μm^2) was analyzed. The scanning electron micrographs were used to count the number of Cp per tenocyte. Measurement of the length and thickness of Cp was performed on the transmission electron micrographs using only the Cp for which the whole length, from the cell body to the terminal adherence, was visible. The thickness of each Cp was measured at its proximal, middle and distal positions.

Statistical analysis

The Kruskal-Wallis rank sum test was employed for examining the number of Cp. Variation in the other morphometric aspects among the three age groups was tested by one-way analysis of variance, followed by the Tukey *post-hoc* test for multiple comparisons of means. Statistical significance was determined at $P<0.05$ in all cases.

RESULTS

Structural organization of the digested tendons

Tendons of the 0-, 10- and 20-day-old piglets digested with 6 N HCl for 15 min were processed for scanning electron microscopy. Clear observation of the tenocytes, Cp, and intercellular spaces strongly proved that the digestion with HCl and collagenase could effectively remove the interstitial connective tissue in the tendon of each age group without damaging the tenocytes (Fig. 1A–C). The intercellular spaces between each Cp varied in size. In addition, the peritendineum, the thick connective tissue sheath enclosing the whole tendon, still existed *in situ* (Fig. 1B). This sheath sent its intratendinous trabeculae or endotendineum into the tendon (Fig. 1A–F). Each primary endotendineum also gave off secondary and tertiary branches to encircle each group of tenocytes to form fascicles of different sizes. The Cp of each tenocyte were found to randomly connect either with the endotendineum or with the Cp of the neighboring cells. This pattern was highly consistent with that observed by optical microscopy (Fig. 1D–F).

Tenocyte population

The digested tendons of all age groups were processed for optical microscopy to analyze the fascicle area, number of cells per fascicle, and cell density (Fig. 1C–F). Although the fascicle areas and cell number per fascicle increased, the cell density decreased with increasing age (Fig. 3, Table 1). Significant differences ($P<0.05$) in each of these three morphometric aspects were found among the three age groups.
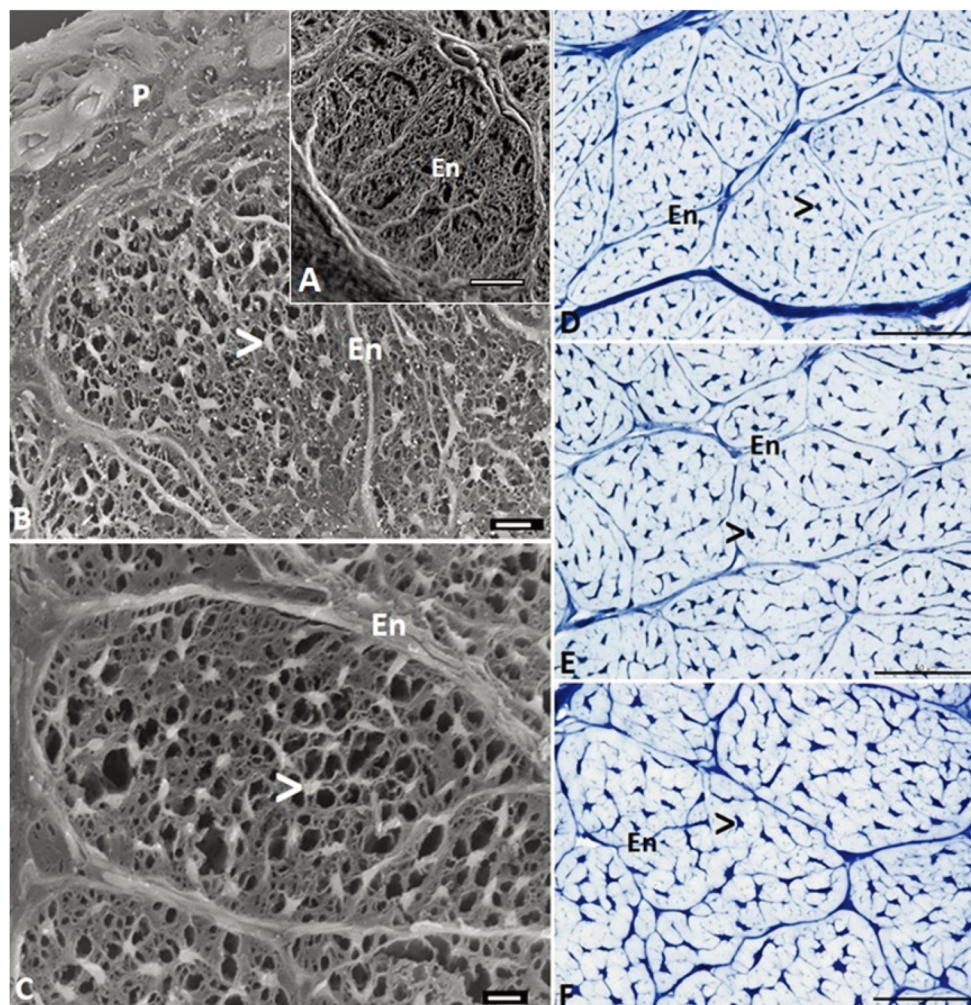
Cp of tenocytes

After digestion, the tendons of all age groups were processed for electron microscopy. In general, the tenocytes displayed an elongated shape, and the elongated nucleus usually occupied almost the entire perikaryon. Cp of different lengths and thicknesses were found extending from all sides of the cell (Fig. 2A and 2B). Two different adhering destinations of Cp were observed, either to the collagen fibrils of the vicinal endotendineum (Cp-to-endotendineum pattern, Fig. 2A) or to the Cp of the adjacent tenocyte (Cp-to-Cp pattern, Fig. 2B). In the Cp-to-endotendineum pattern, numerous finger-like projections were seen emanating from the terminal Cp to adhere to each collagen fibril of the endotendineum (Fig. 2A inset). Adherences were detected not only with the superficial fibrils but also with the collagen fibrils residing inside. These projections spanned approximately 600–700 nm along the longitudinal axis of the collagen fibrils. Adherence in the Cp-to-Cp pattern could appear either in a side-to-side (data not shown) or end-to-end fashion (Fig. 2B inset). Slight expansion at the terminal ends of both Cp was evident. The two Cp adhered to each other by an intercellular junction.

The number of Cp per cell varied from 2 to 6 in the 0-day-old group and from 2 to 7 in the other two groups. Although the distribution of the number of Cp per cell showed slight variation among groups, it did not appear to increase with age (Fig. 4). Indeed, there was no significant difference in the number of Cp per cell among groups ($P=0.545$, Table 1).

The thickness of each Cp was measured perpendicularly at its proximal, middle and distal portions, and the average values of the three sites are presented. The thickness of the Cp ranged from 17.25 to 80.93 nm (with the majority found to be in the range of 30–70 nm) in the 0-day-old piglets, from 31.91 to 83.81 nm (with the majority being 30–60 nm) in the 10-day-old piglets, and from 34.41 to 93.55 nm (with the majority being 30–80 nm) in the 20-day-old piglets (Fig. 4). Although the distribution of the thickness of Cp was slightly shifted to the right, indicating greater thickness with increased age, there was no significant difference in the thickness of Cp among groups ($P=0.105$, Table 1).

The length of each Cp was measured from the initial cytoplasmic protrusion at the perikaryon to its end at the adhering terminal. This length varied from 1.65 to 10.20, 2.70 to 15.35 and 3.78 to 19.39 μm in the 0-, 10- and 20-day-old piglets, respectively (Fig. 4).

Fig. 1. Scanning electron (A–C) and optical (D–F) microscopy images of the superficial digital flexor tendon of 0- (A, D), 10- (B, E), and 20-day-old (C, F) piglets after removal of the intercellular connective tissue. Random ramifications of cytoplasmic processes with intercellular spaces of different sizes were clearly observed. Tenocytes (arrowheads) are enclosed in groups of fascicles of different sizes extending from the peritendineum (P), endotendineum (En), and their secondary to tertiary branches. The intercellular distances varied among different areas, but were generally widened with increasing age [Bar=50 μm (A), 10 μm (B, C), 0.1 mm (D–F)].

The distribution of Cp length was markedly shifted to the right in the older piglets, indicating that the length of Cp increased with increasing age. Indeed, significant differences in the length of the Cp were found among groups ($P<0.05$, Table 1).
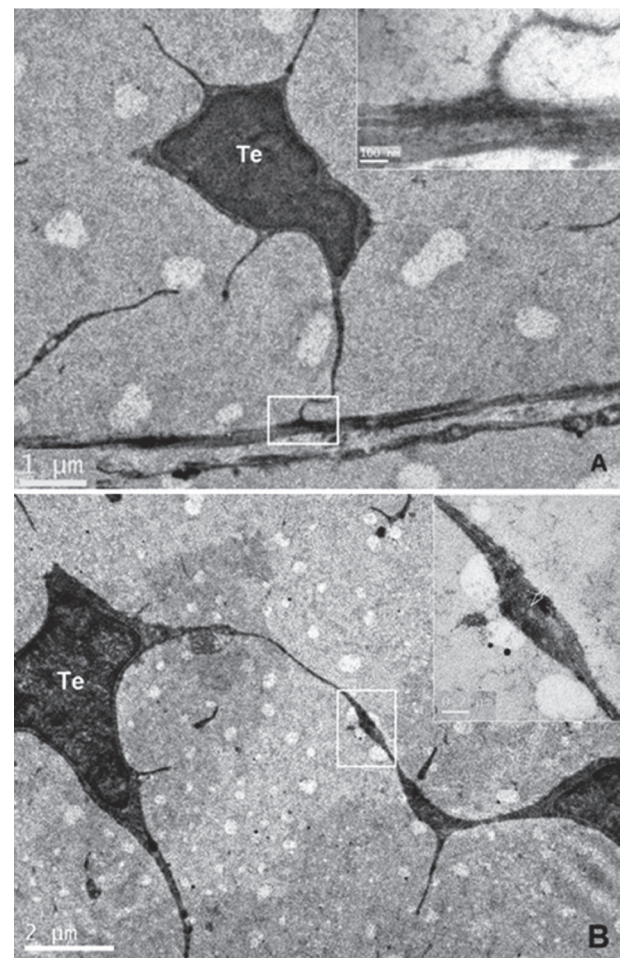
DISCUSSION

The superficial digital flexor muscle of the forelimb in piglets originates from the medial epicondyle at the distal end of the humerus. It is composed of two heads of different sizes. The SDFT originates from the smaller head that descends along the caudomedial side to the ulna and along the caudal side to the metacarpus. At the distal end of the metacarpus, the SDFT divides into medial and lateral branches, which run to insert onto the palmar surface of the middle phalangeal bones. In the present study, only the metacarpal portion of the SDFT of piglets was used for morphometric analysis. In general, the whole tendon is ensheathed by the peritendineum, a thick connective tissue sheath that sends its endotendineum or intratendinous trabeculae into the tendon. The strands of this primary connective tissue send their secondary and perhaps tertiary branches throughout the tendon in a random fashion to form loops surrounding the group of tenocytes. Each loop, the so-called fascicles, contain numerous tenocytes, dense connective tissue, and amorphous ground substances. The aim of this study was to investigate only the tenocytes; thus, connective tissue digestion was applied. The results of the digestion were similar to those reported for the rabbit calcaneal tendon and rat tail tendon [3, 6, 7, 15]. Moreover, HCl, KOH and NaOH, either used alone or in combination with collagenase, have also been reported to have the capability to digest the intercellular connective tissue in the tubular organs and ventricle of rats and humans [11, 26]. Papain was also used for the isolation of collagen and proteoglycans in the human rotator cuff tendon [19]. These acid or basic treatments split the triple helical strands to break down the collagen fibrils into short dialyzable peptides, which are

then further degraded into hydrolysable fragments by relevant enzymes [14]. Collagenase does not affect the tenocytes since the membrane of the cell does not contain collagen. Moreover, the dense and regular arrangement of the endotendineum and peritendineum should play a protective role against the digestion by collagenase. Thus, the intact tenocytes and collagenous trabeculae observed in the whole tendon indicated that the samples were well-prepared for the morphometric analysis (Fig. 1A–C).

Comparison between the digested tendons of 0-, 10- and 20-day-old piglets using optical microscopy clearly revealed that the tenocyte number per fascicle, intercellular spans between each tenocyte, and fascicle area were significantly increased with increasing age (Table 1, Fig. 1C–F). In younger animals, an increased number of tenocytes and amount of the ECM of the growing tendon truly reflect the active mitotic activity of the tenocytic precursor cells, which subsequently transform to tenocytes to produce the ECM in order to cope with the increasing area of each fascicle [5, 8, 16, 25]. In the present study, the total numbers of tenocytes changed significantly with age but still dispersed within the growing fascicles. The increase in the amount of intercellular connective tissue and amorphous ground substances could certainly widen the intercellular space, thereby resulting in the observed increase of the fascicle area. Although the number of tenocytes shows a marked increase in young animals, the rate of increase might not be constant throughout the animal's life. Accordingly, Lavagnino *et al.* [10] demonstrated a significant decrease in this number in older rats. Moreover, the non-proportional increase between cell number and fascicle area would result in a diminishing outcome with respect to the cell density (Table 1). Although similar results were reported by Ippolito *et al.* [7], Lavagnino *et al.* [10], Nakagawa *et al.* [17] and Stanley *et al.* [24], different results were demonstrated in other studies, including an increase [18, 24] and no change in cell density [4, 27]. The likely sources of these conflicting findings are different quantification methods, ages of the animals, and treatments in the different studies.
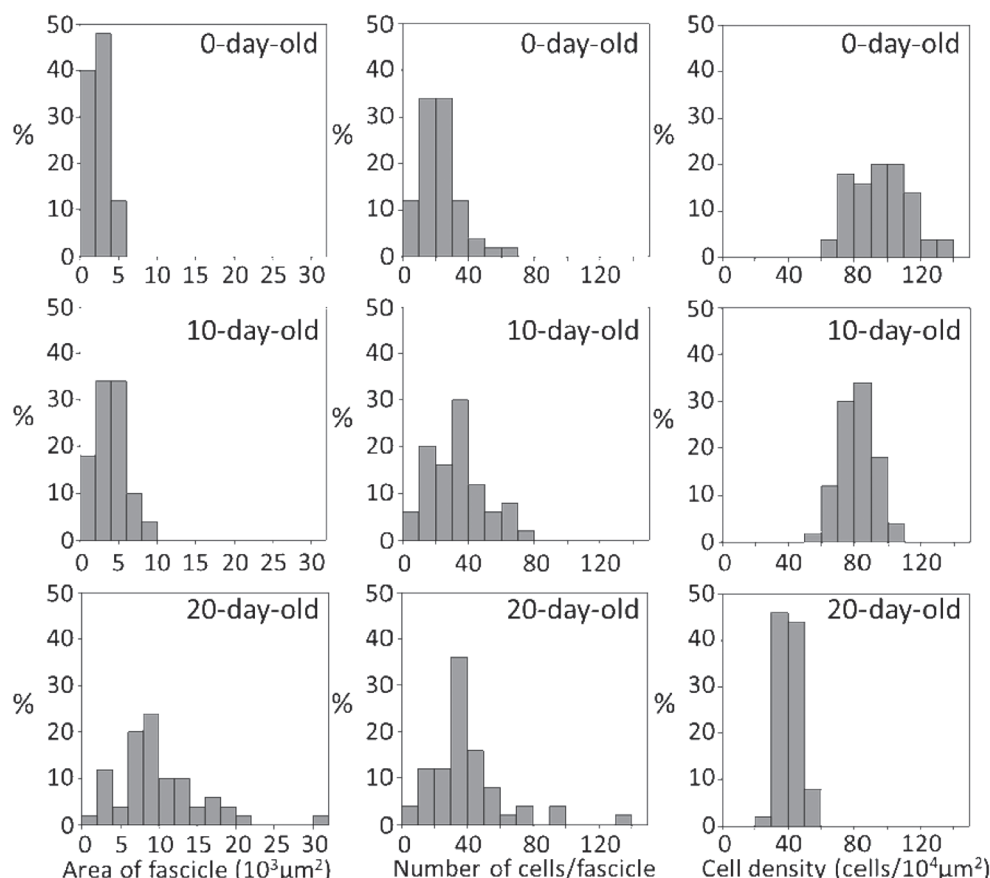
Our ultrastructural investigation did not reveal a substantial difference in the morphology of tenocytes and their Cp in the piglet tendons. The shape and growth of the tenocyte followed the direction of the running course of the intercellular connective tissue, as found in other studies [8, 15]. Although the number of Cp per tenocyte did not differ significantly among the age groups in the present study, Murata *et al.* [15] reported a reduction of the secondary branches of the Cp in the calcaneal tendon of older rats. These branches frequently appeared with fragmentations or perforations, especially at their ends. However, similar to Murata

Fig. 2. Transmission electron micrographs of the superficial digital flexor tendon of 0-day-old piglets demonstrating two different adhering patterns of the cytoplasmic processes (Cp) of tenocytes (Te): the Cp-to-endotendineum (A) and Cp-to-Cp pattern (B). Cp of different lengths extended from all sides of the tenocytes. Insets in A and B showed enlargement of the rectangles in their corresponding micrographs. At the endotendineum (A inset), the Cp expanded and sent its finger-like projections to adhere with the collagen fibrils. Slight expansion was detected at the terminal ends of both Cp in the Cp-to-Cp pattern. The intercellular junction was also evident (B inset, arrow) [Bar=1 μm (A), 100 nm (A inset), 2 μm (B) and 0.2 μm (B inset)].

et al. [15], we observed elongation of the primary Cp of the tenocyte. The increase in Cp length appeared to correspond with the increase of fascicle area. Since the tenocytes in young animals actively produce the ECM, the distance between each cell would likely increase as a result of the increased amount of ECM [8, 25], which would in turn increase the fascicle area. Thus, we suggest that the length of Cp increased to conform to the increased fascicle area, because the tenocyte had to increase its Cp length to be able to span across the enlarged space [8]. Moreover, the increased fibrous and non-fibrous components [8] should be able to yield a certain compressive effect against the thickening process of the lengthened Cp. Accordingly, there was no significant difference in the thickness of Cp among the tendons of piglets of different ages.

Adherence to the terminal end of each Cp appeared in two different patterns: Cp-to-Cp and Cp-to-endotendineum. The Cp-to-Cp pattern is the adherence of a Cp of one tenocyte to a Cp of the adjacent tenocyte where intercellular communication takes place. The gap junction located in this intercellular structure should be necessary for the cellular coordinate for proliferation, especially in the growing tendon [1, 12, 23, 24]. In the Cp-to-endotendineum pattern, the functional tenocyte extends its Cp across the intercellular space to anchor or hold onto the vicinal intratendinous trabeculae. From a morphological standpoint, the adherence of the Cp-to-endotendineum pattern seems to be more secure than that of the Cp-to-Cp pattern. The finger-like projections allow one Cp to adhere to many superficial and deep collagen fibrils. In addition, the span of the adherence of a Cp covers a larger area of the

Fig. 3. Distribution of the fascicle area, number of cells per fascicle, and cell density in the superficial digital flexor tendon of 0-, 10- and 20-day-old piglets. The fascicle area and cell number increased, whereas the cell density decreased with age. However, there was only a slight increase in the number of cells per fascicle.
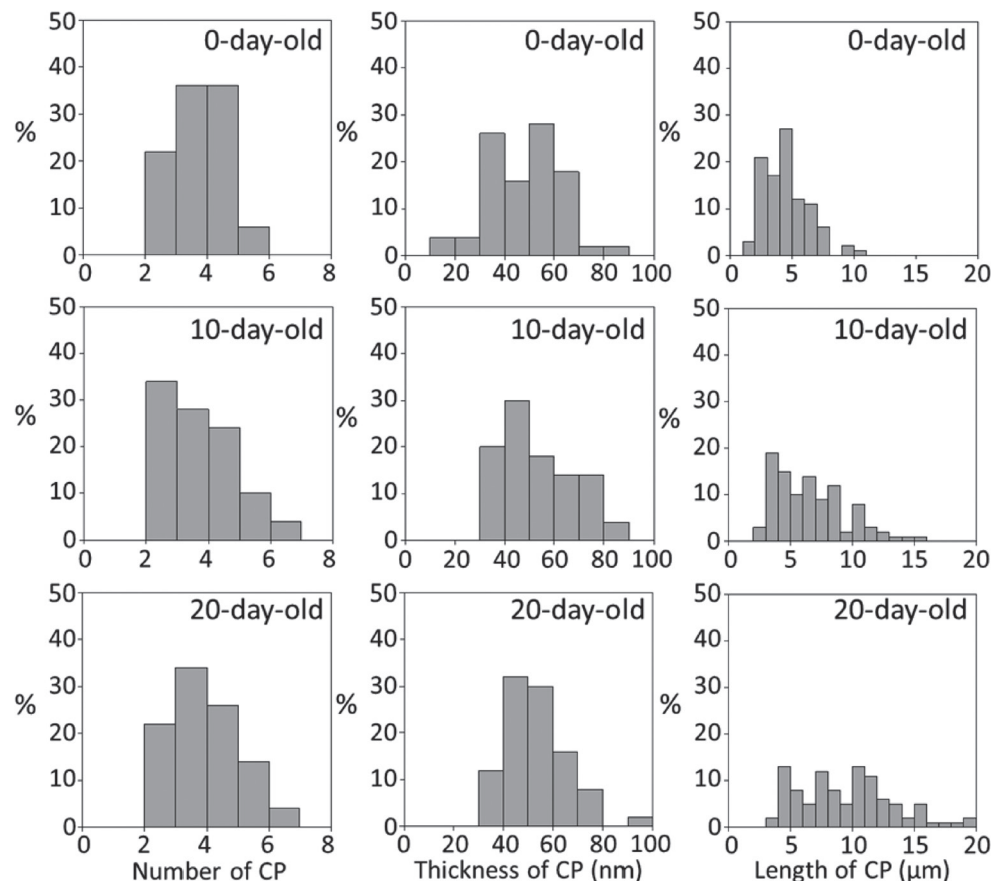
Table 1. Morphometric analyses of the tendons of 0- (0 d), 10- (10 d) and 20-day-old (20 d) piglets

Morphometry (Mean ± SE)	0 d	10 d	20 d
Area of fascicle (μm^2)	2,428.9 ± 158.42[a]	4,057.5 ± 264.36[b]	9,897.9 ± 775.29[c]
Number of cells/fascicle	23.1 ± 1.62[a]	33.7 ± 2.36[b]	40.1 ± 3.35[c]
Cell density (/10^4 μm^2)	96.7 ± 2.42[a]	81.6 ± 1.54[b]	40.9 ± 0.89[c]
Number of Cp/cell	4.3 ± 0.12[a]	4.2 ± 0.16[a]	4.4 ± 0.16[a]
Thickness of Cp (nm)	48.1 ± 1.99[a]	53.4 ± 2.06[a]	53.0 ± 1.75[a]
Length of Cp (μm)	4.5 ± 0.18[a]	6.6 ± 0.28[b]	9.5 ± 0.38[c]

Different superscripts in the same row indicate statistical significance at $P<0.05$. Cp, cytoplasmic processes.

endotendineum. Therefore, this adherence should function to conserve the cellular stabilization and integrity of the tendon.

In summary, an increase in length and diameter of the SDFT in growing piglets likely results from the increase in the tenocyte number per fascicle and fascicle area. The decreased cell density results from the disproportional increase between the tenocyte number and fascicle area. The length of the Cp was increased in correspondence with the expanding intercellular space, together with the increasing fascicle area. However, no difference was found in the number of Cp per cell and the thickness of Cp among age groups. These morphometric characteristics correlate with the growth of the tendon to some extent. Further study on these particular aspects, for example using serial block face-scanning electron microscopy [25], would help to gain a deeper understanding of the growing pattern and provide valuable information for three-dimensional reconstruction of the tenocytes, intercellular tissues, and tendon as a whole.

Fig. 4. Number of cytoplasmic processes (Cp) per cell, thickness of Cp, and length of Cp in the superficial digital flexor tendon of 0-, 10- and 20-day-old piglets.

REFERENCES

1. Becker, D. L. and Mobbs, P. 1999. Connexin alpha1 and cell proliferation in the developing chick retina. *Exp. Neurol.* **156**: 326–332. [Medline] [CrossRef]
2. Benjamin, M. and Ralphs, J. R. 1997. Tendons and ligaments--an overview. *Histol. Histopathol.* **12**: 1135–1144. [Medline]
3. Coffey, J. W., Fiedler-Nagy, C., Georgiadis, A. G. and Salvador, R. A. 1976. Digestion of native collagen, denatured collagen, and collagen fragments by extracts of rat liver lysosomes. *J. Biol. Chem.* **251**: 5280–5282. [Medline]
4. Couppé, C., Svensson, R. B., Heinemeier, K. M., Thomsen, E. W., Bayer, M. L., Christensen, L., Kjær, M., Magnusson, S. P. and Schjerling, P. 2017. Quantification of cell density in rat Achilles tendon: development and application of a new method. *Histochem. Cell. Biol.* **147**: 97–102. [Medline] [CrossRef].
5. Dowling, B. A., Dart, A. J., Hodgson, D. R. and Smith, R. K. 2000. Superficial digital flexor tendonitis in the horse. *Equine Vet. J.* **32**: 369–378. [Medline] [CrossRef]
6. Evan, A. P., Dail, W. G., Dammrose, D. and Palmer, C. 1976. Scanning electron microscopy of cell surfaces following removal of extracellular material. *Anat. Rec.* **185**: 433–445. [Medline] [CrossRef]
7. Ippolito, E., Natali, P. G., Postacchini, F., Accinni, L. and De Martino, C. 1980. Morphological, immunochemical, and biochemical study of rabbit achilles tendon at various ages. *J. Bone Joint Surg. Am.* **62**: 583–598. [Medline] [CrossRef]
8. Kalson, N. S., Lu, Y., Taylor, S. H., Starborg, T., Holmes, D. F. and Kadler, K. E. 2015. A structure-based extracellular matrix expansion mechanism of fibrous tissue growth. *eLife* **4**: e05958. [Medline] [CrossRef]
9. Kannus, P. 2000. Structure of the tendon connective tissue. *Scand. J. Med. Sci. Sports* **10**: 312–320. [Medline] [CrossRef]
10. Lavagnino, M., Gardner, K. and Arnoczky, S. P. 2013. Age-related changes in the cellular, mechanical, and contractile properties of rat tail tendons. *Connect. Tissue Res.* **54**: 70–75. [Medline] [CrossRef]
11. Macchiarelli, G. and Ohtani, O. 2001. Endomysium in left ventricle. *Heart* **86**: 416. [Medline] [CrossRef]
12. Maeda, E., Ye, S., Wang, W., Bader, D. L., Knight, M. M. and Lee, D. A. 2011. Gap junction permeability between tenocytes within tendon fascicles is suppressed by tensile loading. *Biomech. Model. Mechanobiol.* **11**: 439–447. [Medline] [CrossRef]
13. Magnusson, S. P., Hansen, P. and Kjaer, M. 2003. Tendon properties in relation to muscular activity and physical training. *Scand. J. Med. Sci. Sports* **13**: 211–223. [Medline] [CrossRef]
14. Mookhtiar, K. A. and Van Wart, H. E. 1992. *Clostridium histolyticum* collagenases: a new look at some old enzymes. *Matrix Suppl.* **1**: 116–126. [Medline]
15. Murata, H., Nishizono, H. and Miyoshi, M. 2000. Postnatal development of structure and arrangement of tendon cells. A scanning and transmission electron microscope study in the rat calcaneal tendon. *Okajimas Folia Anat. Jpn.* **77**: 77–86. [Medline] [CrossRef]
16. Nagy, I. Z., Von Hahn, H. P. and Verzár, F. 1969. Age-related alterations in the cell nuclei and the DNA content of rat tail tendon. *Gerontologia* **15**:

258–264. [Medline] [CrossRef]

17. Nakagawa, Y., Majima, T. and Nagashima, K. 1994. Effect of ageing on ultrastructure of slow and fast skeletal muscle tendon in rabbit Achilles tendons. *Acta Physiol. Scand.* **152**: 307–313. [Medline] [CrossRef]

18. Pingel, J., Lu, Y., Starborg, T., Fredberg, U., Langberg, H., Nedergaard, A., Weis, M., Eyre, D., Kjaer, M. and Kadler, K. E. 2014. 3-D ultrastructure and collagen composition of healthy and overloaded human tendon: evidence of tenocyte and matrix buckling. *J. Anat.* **224**: 548–555. [Medline] [CrossRef]

19. Riley, G. P., Harrall, R. L., Constant, C. R., Chard, M. D., Cawston, T. E. and Hazleman, B. L. 1994. Tendon degeneration and chronic shoulder pain: changes in the collagen composition of the human rotator cuff tendons in rotator cuff tendinitis. *Ann. Rheum. Dis.* **53**: 359–366. [Medline] [CrossRef]

20. Roberts, T. J., Marsh, R. L., Weyand, P. G. and Taylor, C. R. 1997. Muscular force in running turkeys: the economy of minimizing work. *Science* **275**: 1113–1115. [Medline] [CrossRef]

21. Squier, C. A. and Bausch, W. H. 1984. Three-dimensional organization of fibroblasts and collagen fibrils in rat tail tendon. *Cell Tissue Res.* **238**: 319–327. [Medline] [CrossRef]

22. Squier, C. A. and Magnes, C. 1983. Spatial relationships between fibroblasts during the growth of rat-tail tendon. *Cell Tissue Res.* **234**: 17–29. [Medline] [CrossRef]

23. Stanley, R. L., Fleck, R. A., Patterson-Kane, J. C., Goodship, A. E. and Ralphs, J. R. 2006. Confocal microscopy and image analysis of connexin plaques in equine tendon. *Microsc. Analys.* **3**: 5–6.

24. Stanley, R. L., Fleck, R. A., Becker, D. L., Goodship, A. E., Ralphs, J. R. and Patterson-Kane, J. C. 2007. Gap junction protein expression and cellularity: comparison of immature and adult equine digital tendons. *J. Anat.* **211**: 325–334. [Medline] [CrossRef]

25. Starborg, T., Kalson, N. S., Lu, Y., Mironov, A., Cootes, T. F., Holmes, D. F. and Kadler, K. E. 2013. Using transmission electron microscopy and 3View to determine collagen fibril size and three-dimensional organization. *Nat. Protoc.* **8**: 1433–1448. [Medline] [CrossRef]

26. Ushiki, T. and Murakumo, M. 1991. Scanning electron microscopic studies of tissue elastin components exposed by a KOH-collagenase or simple KOH digestion method. *Arch. Histol. Cytol.* **54**: 427–436. [Medline] [CrossRef]

27. Wood, L. K. and Brooks, S. V. 2016. Ten weeks of treadmill running decreases stiffness and increases collagen turnover in tendons of old mice. *J. Orthop. Res.* **34**: 346–353. [Medline] [CrossRef]