Histol Histopathol (2013) 28: 623-632
DOI: 10.14670/HH-28.623

http://www.hh.um.es

Histology and Histopathology
Cellular and Molecular Biology

The plantaris tendon in association with mid-portion Achilles tendinosis – Tendinosis-like morphological features and presence of a non-neuronal cholinergic system

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Summary. The plantaris tendon is often neglected in morphological/clinical studies on the lower extremity. There is, however, clinical evidence that the plantaris tendon is involved in cases with Achilles midportion tendinopathy/tendinosis. It is nevertheless unclear if the plantaris tendon exhibits tendinosis-like features in this situation. We therefore investigated the plantaris tendon of patients with midportion Achilles tendinosis when the plantaris tendon was found to be located very close to or invaginated into the Achilles tendon, a situation which very often has been found to be the case. There was a very large number of tenocytes in the tendon tissue and the tenocytes showed abnormal and irregular appearances, exhibiting widened/rounded and wavy appearances, and were frequently lined up in rows. These features are characteristic features in Achilles tendinosis tendons. The tendon cells showed a distinct immunoreaction for the acetylcholine (ACh) -producing enzyme choline acetyltransferase (ChAT). Frequent fibroblasts were found in the loose connective tissue and these cells also showed a marked ChAT immunoreaction. The study shows that the plantaris tendon is morphologically affected in a similar way to the Achilles tendon in cases with midportion Achilles tendinosis and medial pain. The plantaris tendon may accordingly be a co-factor in these cases. The results also favour that there is a local ACh production both within the tendon tissue of the plantaris tendon and in the loose connective tissue. In conclusion, it is evident that plantaris tendons lying invaginated into or very close to the Achilles tendon in cases with midportion Achilles tendinosis show similar tendinosis features as previously shown for the Achilles tendon itself in these cases.

Key words: Plantaris tendon, Achilles tendon, Tendinopathy, Tenocyte morphology, Cholinergic, Choline acetyltransferase

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Introduction

Chronic tendon pain is a condition affecting many tendons in the body. One of the most afflicted is the Achilles tendon. The portion that is particularly affected is the midportion. Although currently used treatment methods have been usually successful, there are still cases with midportion Achilles tendinopathy (tendinosis) that have not been found to be curable (Alfredson, 2011a). When re-operating on cases where the ordinary treatment procedure failed, it was found that several (5 out of 7) of these had an invaginated plantaris tendon in the ventromedial aspect of the Achilles tendon (Alfredson, 2011b). During Achilles tendoscopy, it has also been noted that the plantaris tendon is affixed to the medial side of the Achilles tendon in cases with tendinopathy (van Sterkenburg et al., 2011). In another recent study, an invaginated, or "close by located", enlarged plantaris tendon was found in 58/73 (80%) midportion Achilles tendinosis tendons undergoing surgical treatment with ultrasound+Doppler-guided scraping (Alfredson, 2011b). Interestingly, clinical outcomes in response to excision of the plantaris tendon in cases with Achilles tendinosis have been good (Alfredson, 2011b; van Sterkenburg and van Dijk, 2011). Although there are findings which suggest that the plantaris tendon is afflicted in cases with Achilles

tendinosis, there is no morphological evidence favouring this. No morphological study on the plantaris tendon in these cases has thus been performed. Concerning the Achilles tendon, classic morphological features have been noted for the tendon tissue in cases with tendinosis. These include the presence of a large number of tenocytes and marked changes in tenocyte morphology (Astrom and Rausing, 1995; Movin et al., 1997; Khan et al., 1999; Maffulli et al., 2004; Shalabi, 2004; Bjur et al., 2005, 2008). Characteristic tenocyte changes are widening/rounding, an occurrence of a wavy appearance and lining-up of widened/rounded tenocytes. It is not known if there are similar morpohologial features in the plantaris tendon when found in close relationship to the thickened midportion Achilles tendinosis tendon.

Recently, it has also been observed that the tenocytes of Achilles tendinosis tendons display expressions of enzymes/transporters showing that they can produce nerve signal substances; such features were infrequently observed in pain-free Achilles tendons (Bjur et al., 2008; Scott et al., 2008). One such nerve signal substance is acetylcholine (Bjur et al., 2008). There is no information as to whether the tenocytes in the plantaris tendon show such features.

In the present study, the plantaris tendon of 17 patients with midportion Achilles tendinopathy/tendinosis was investigated morphologically and with respect to ChAT immunoreaction patterns. The patients complained of having midportion Achilles tendon pain, most often on the medial side. The plantaris tendon was released and excised in all these patients.

Materials and methods

Individuals

Seventeen patients who had had a long duration (>3 months) of pain symptoms, and ultrasound+Doppler verified changes in the Achilles midportion diagnosed as tendinosis were included in this study. Ultrasound+Doppler examination showed thickening, irregular tendon structure, hypo-echoic regions, and localized high blood flow outside and inside the ventral midportion. Ten of the patients were men with a mean age of 40.5 years and a range from 18 to 53 years, and 7 were women with a mean age of 54,7 years and a range from 50 to 60 years. Specimens from the Achilles tendon of 2 of these patients were also evaluated in parallel. Furthermore, the plantaris tendon from an individual without any known history of Achilles tendon pain was included.

Sampling

The surgical procedure was done under local anaesthesia (Pilokainhydrochloride 4-5 ml, 10 mg/ml, Astra Zeneca, Södertälje, Sweden). Through a short longitudinal skin incision on the medial side, the Achilles tendon was visualized. In all patients, a plantaris tendon, often enlarged, was found very close to the ventral and medial Achilles tendon. The plantaris tendon was sometimes found to be invaginated into the Achilles tendon. Most often, fat tissue was found in the loose peritendinous connective tissue that was interpositioned between the Achilles and plantaris tendons. The plantaris tendon was carefully released, followed distally and proximally, and cut at both ends. The ventral Achilles tendon was "scraped" in the regions with high blood flow, according to currently outlined procedures (Alfredson, 2011b).

The plantaris tendons from all patients, and biopsies from the ventro-medial Achilles tendon in two of the patients, were collected (c.f. above). Furthermore, a plantaris tendon from an individual (female; 27 years of age) without any known history of Achilles tendon pain and for whom ultrasound+Doppler examination had revealed no pathological features was included in the studies. Also in this case, a short longitudinal skin incision on the medial side was made under local anaestesia (c.f. above) and the medial Achilles tendon and the plantaris tendon were visualized. The plantaris tendon lay close to the medial Achilles midportion. Due to ethical reasons, we restricted the obtaining of normal plantaris tendon to this case.

The study protocol was approved by the Regional Ethical Board in Umeå (dnr 04-157M; 2011-83-32M). The experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Fixation and sectioning

Immediately after the tendon samples were taken, they were transported to a nearby located laboratory, and specimens from the samples were fixed overnight at 4°C in a solution of 4% formaldehyde in 0.1M phosphate buffer, pH 7.0. The specimens were thereafter thoroughly washed in Tyrode's solution containing 10% sucrose, at 4°C overnight, mounted on thin cardboard in OCT embedding medium (Miles Laboratories, Naperville, IL, USA) and frozen at -80°C until sectioning. A series of 7-8 μm thick sections were cut using a cryostat (Leica Microsystem CM 3000, Heidelberg, Germany). The sections were mounted on slides pre-coated with crome-alum gelatine, dried, and processed for immunohistochemistry, or stained with haematoxylin-eosin in order to explore tissue morphology.

Other specimens from the samples were processed in a chemically unfixed state. These were directly frozen and thereafter sectioned as described above. Sections of these were processed for haematoxylin-eosin.

Immunofluorescence processing

Immunostaining for ChAT

Immunostaining for demonstration of ChAT immunoreactions was performed. The procedures were

as previously described (Bjur et al., 2008) with the exception that Alexa FluorO 488 donkey antigoat IgG (Invitrogen, dilution 1:300) was used as secondary antiserum. Examination was carried out in a Zeiss Axioscope 2 plus microscope equipped with epi-fluorescence technique and an Olympus DP70 digital camera.

The ChAT antibody utilized was raised in goat (AB 144P), obtained from Chemicon Temecula, USA, and used at a dilution of 1:50. The immunogen for this antibody is human placental ChAT enzyme. The antibody is reported by the supplier to be reactive with ChAT in a number of mammalian species, including humans. For control purposes, the ChAT antiserum was preincubated overnight at 4°C with its corresponding antigen (AG220, Chemicon; 20 μg/ml antiserum). Control staining also included staining when PBS was used instead of the primary antibody.

Immunostainings for markers for fibroblasts and white blood cells

Immunostainings were used for the demonstration of certain white blood cell markers and fibroblasts. All that were used were mouse monoclonals. One corresponded to a macrophage (CD68) antibody (M0814), and was from DakoCytomation (Glostrup, Denmark), and another was a T-cell and neutrophils antibody (MCA805G) from AbD Serotec (Oxford, UK). The dilutions used for these were in both cases 1:50. An antibody against fibroblasts from DakoCytomation (Glostrup, Denmark) (M0877; anti-human fibroblast clone 5B5, dilution used 1:100) was furthermore applied. This antibody is described to be useful for the identification of actively collagen-synthesizing fibroblasts and fibroblast-like cells. The procedures used for the demonstration of the white blood cell markers were as previously described (Spang et al., 2012), with the exception that another staining variant was also used in parallel, whereby acid potassium permanganate was not used as a preincubation step. The procedures for demonstration of fibroblast marker also corresponed to these procedures. For further details, see (Spang et al., 2012). Control stainings for all staining variants corresponded to stainings when the primary antisera were substituted by PBS/BSA.

Results

Morphology of the plantaris tendons and the peritendinous connective tissue in the cases with Achilles tendinosis

The plantaris tendons of patients operated due to Achilles tendinosis were examined. The tendon tissue of the plantaris tendons was, as has been observed for other tendons, found to be built up of linearly aligned collagen, groups of these being separted by strands of

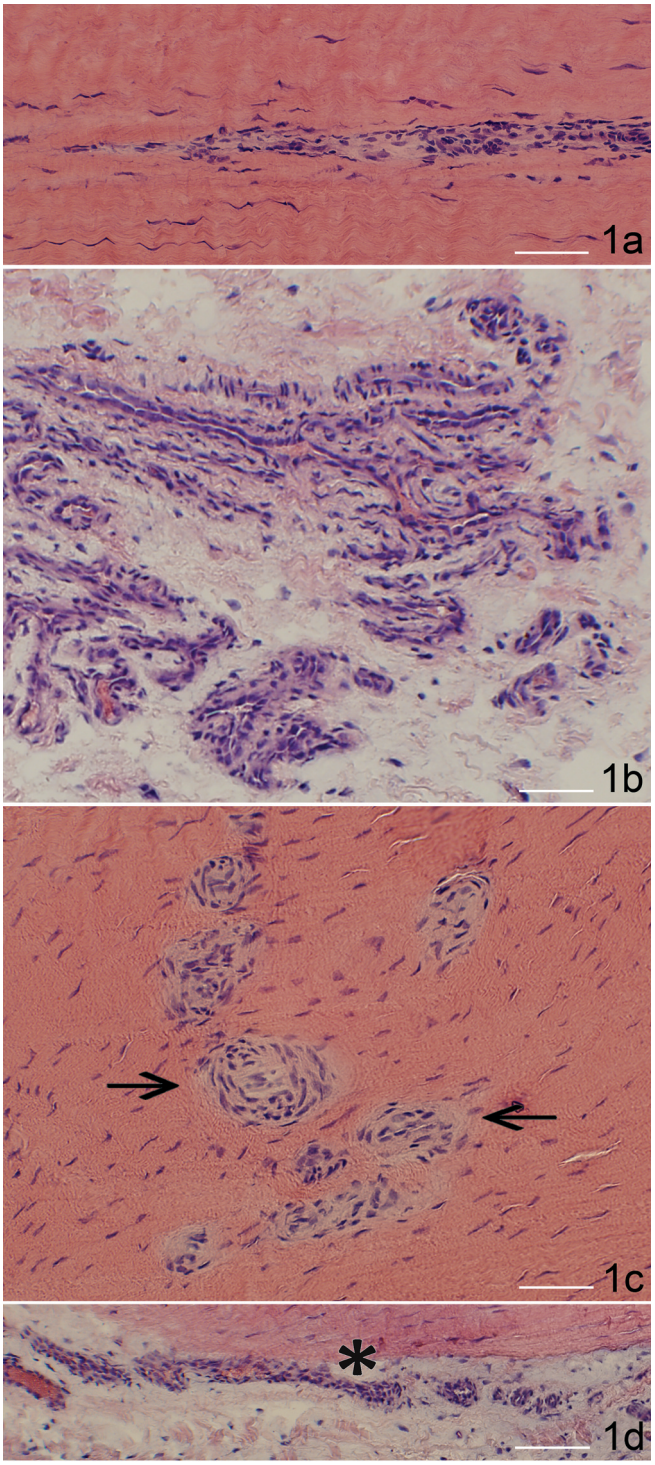

Fig. 1. Plantaris tendon tissue **(a, c)**, adjacent peritendinous connective tissue **(b)** and junction area between these tissues **(d)**. The specimens were from Achilles tendinosis cases. A blood vessel (middle) and tenocytes (above and below) **(a),** and a marked occurrence of fine blood vessels in the peritendinous connective tissue **(b)** are shown. Frequent blood vessels and the occurrence of a large number of tenocytes in the tendon tissue are shown in **(c)** (arrows at blood vessels). There are also blood vessels in the border zone (indicated with asterisk) between tendon tissue and peritendinous connective tissue **(d)**. Bars: 20 μm.

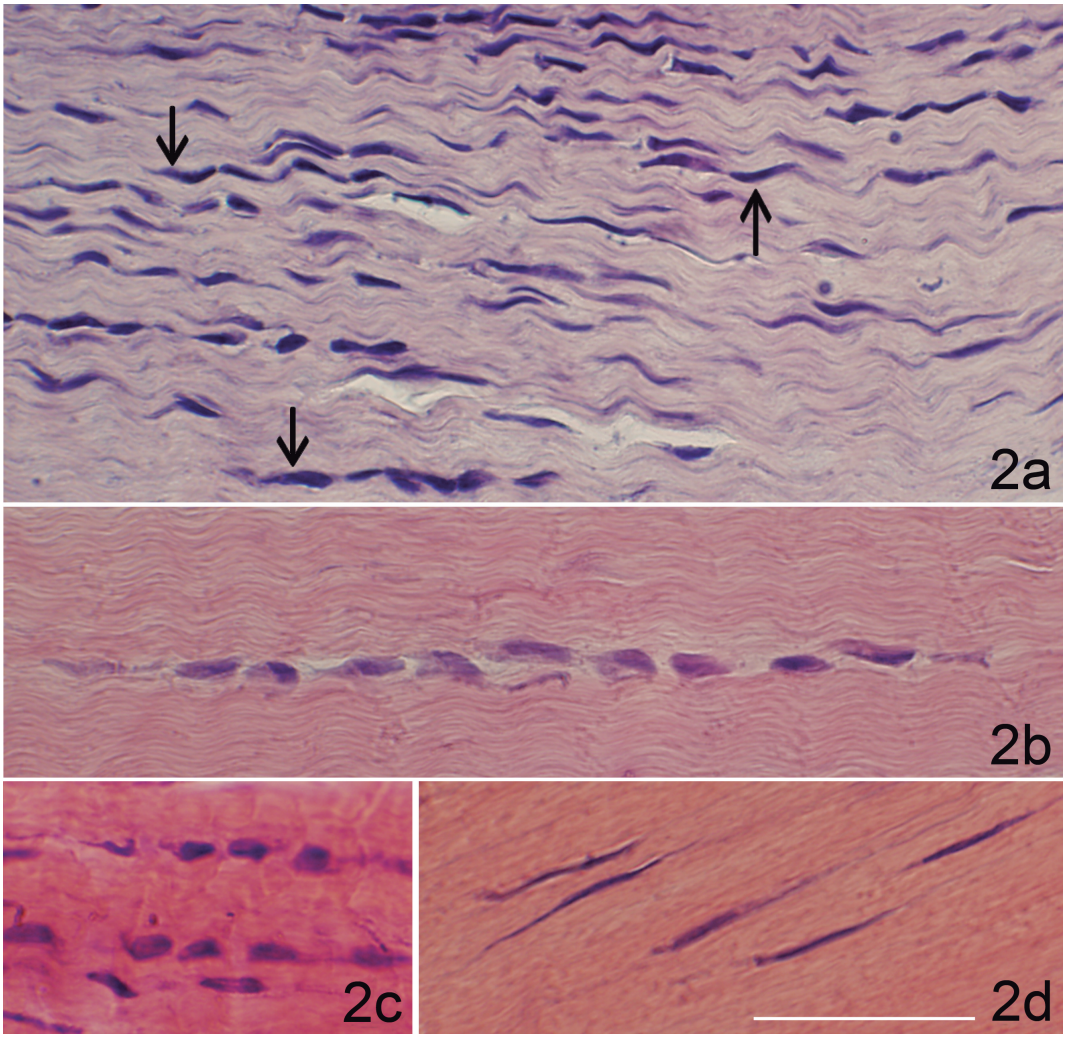

Fig. 2. Plantaris tendon tissue. Achilles tendinosis cases. There is a large number of tenocytes in the tissue as shown by an overview **(a)**, parts of the tenocytes showing a widened appearance (arrows). Tenocytes showing widened appearance are found to be lined up in a row **(b)**. Widened/abnormal appearance of the tenocytes are shown in **(c)**. Occurrence of slender spindle-shaped forms of tenocytes was only sometimes seen **(d)**. Bar: 20 μm.

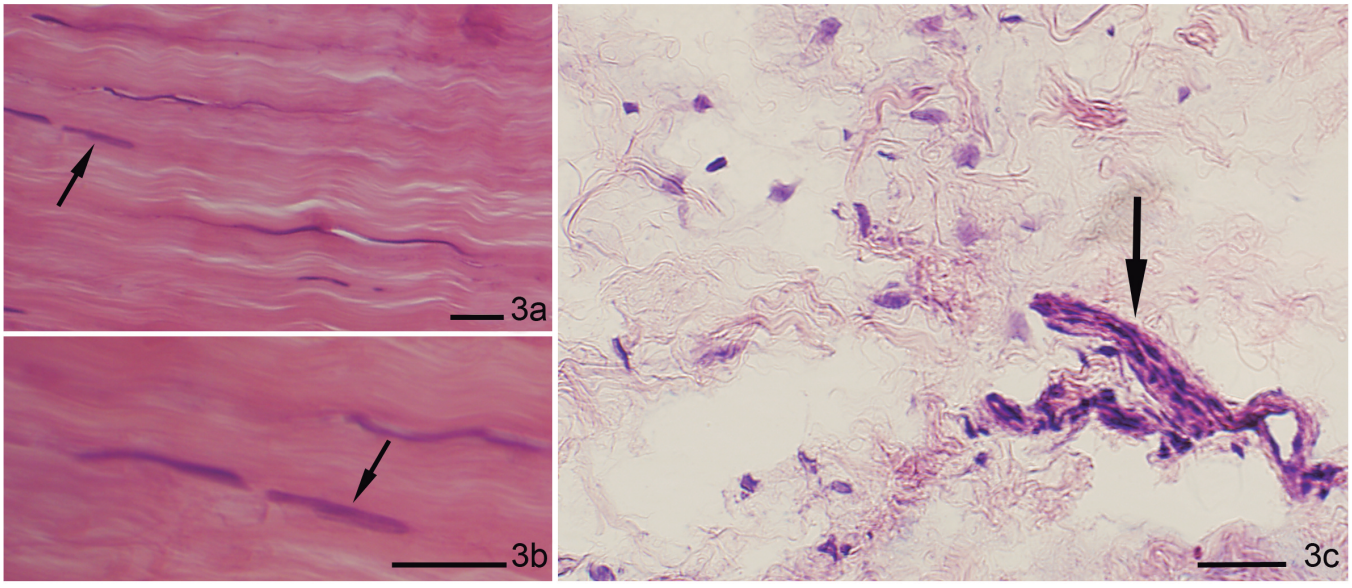

Fig. 3. Plantaris tendon tissue. Normal tendon (case without tendinosis in the Achilles tendon). The occurrence of comparatively few, slender and spindle-shaped tenocyters is seen for tendon tissue **(a)**. Larger magnification in **(b)** (arrow at one of the tenocytes). The occurrence of a small blood vessel (arrow) and a few scattered cells in the loose peritendinous connective tissue are shown in **(c)**. Bars: 20 μm.

connective tissue, and dispersed tenocytes. Blood vessels occurred in the strands (Fig. 1a). Overall, there was a large number of blood vessels within the tendon tissue (Fig. 1c).

The loose peritendinous connective tissue, which is known to be located just outside the tendon tissue of the plantaris tendon, was contained alongside the tendon tissue for some of the specimens obtained. There was also a large number of fine (Fig. 1b) as well as large blood vessels in this tissue. Parts of these blood vessels were directly apposed to the outer parts of the tendon tissue (Fig. 1d). Dispered cells and cells lying in groups were very frequently found in the loose peritendinous connective tissue.

There was a large number of tenocytes within the tendon tissue of the plantaris tendons (Fig. 2a). The tenocytes showed varying appearances. They generally did not show the slender and spindle-shaped form that is characteristic for tenocytes of normal tendon tissue, but showed abnormal and irregular appearances, exhibiting widened/rounded and wavy appearances (Figs. 1a, 2a-d). Some tenocytes were markedly rounded (Fig. 2c). Tenocytes showing widened/rounded appearance could often be seen to be lined up in rows (Fig. 2b). The irregular and abnormal tenocyte appearances were seen all through the specimens. Tenocytes showing spindle-

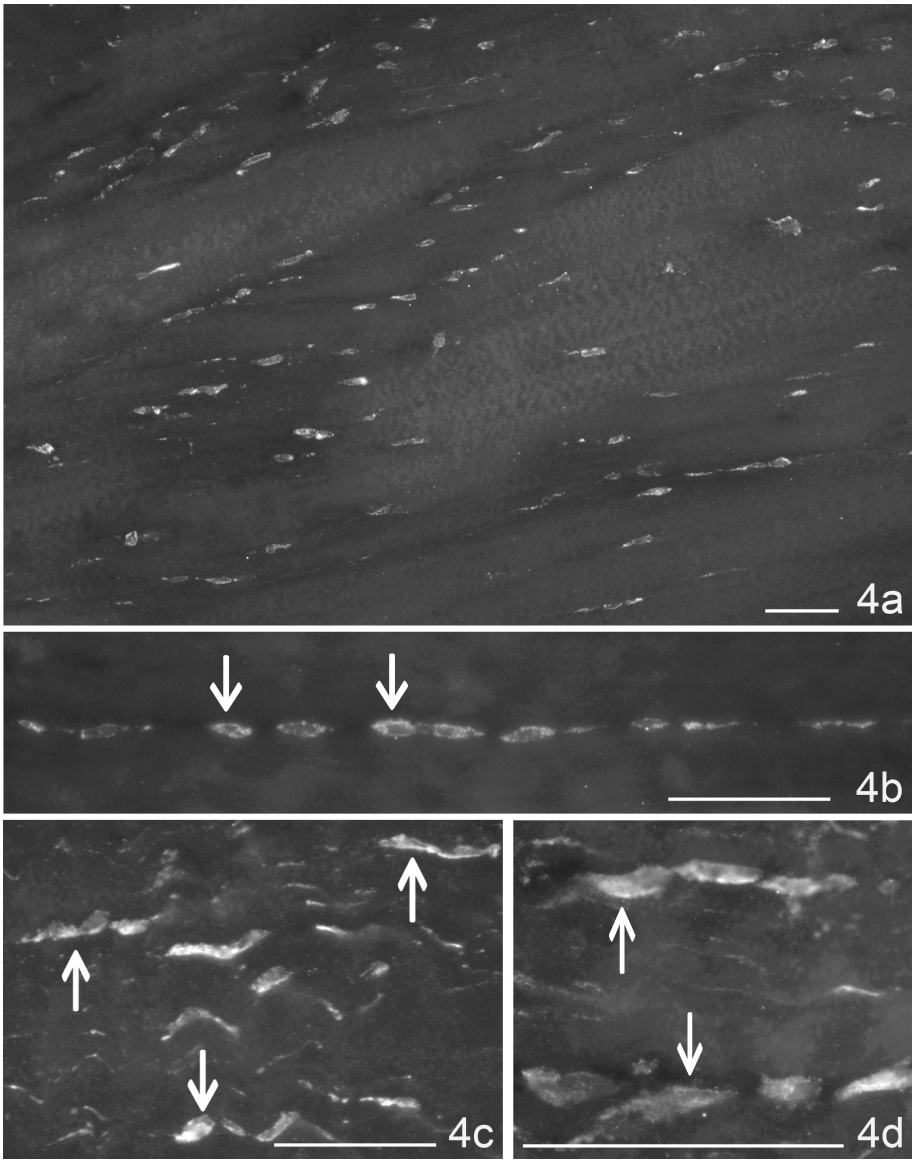

Fig. 4. Immunostaining for demonstration of ChAT. Plantaris tendon tissue. Achilles tendinosis cases. In the overview in **(a)**, it can be seen that the frequently occurring tenocytes show immunoreactions. Immunoreactions in widened/cylindrical tenocytes are shown in **(b)** (arrows). Abnormal tenocytes with immunoreactions are shown in **(c, d)** (arrows at some of the immunoreactive tenocytes). Bars: 20 μm.

shaped and slender forms could, however, sometimes be seen in parts of the specimens (Fig. 2d).

Morphology of the adjacent Achilles tendon

Specimens of two Achilles tendons lying adjacent to the plantaris tendons described above, were also examined. These showed typical tendinosis appearances, including the presence of a large number of tenocytes, an occurrence of irregular and abnormal morphologies for these cells and the presence of lining up of widened/rounded tenocytes (not shown).

Morphology of control plantaris tendon

The morphology of the control plantaris tendon was clearly different from that of the plantaris tendons described above. The tenocytes were comparatively few and showed a straight and spindle-shaped form all through the specimen (Fig. 3a,b). A few blood vessels were seen in the peritendinous connective tissue (Fig. 3c).

Immunohistochemistry

Tendon tissue of plantaris tendons from cases with Achilles tendinosis

Marked ChAT immunostaining was noticed for most of the tenocytes of the plantaris tendons from the cases with Achilles tendinosis. In overview at low magnification, a multitude of immunoreactive tenocytes could thus be seen in the tendon tissue (Fig. 4a). The tenocytes that were lined up in rows were ChAT immunoreactive (Fig. 4b), as was the case for tenocytes showing various abnormal appearances (Fig. 4c,d). The specificity of the reactions was verified via preabsorption stainings (Fig. 5).

There were no specific reactions in the tenocytes after processing for CD68 or after processing for T cell/neutrophil marker (not shown).

Tendon tissue of control plantaris tendon

The tenocytes of the control plantaris tendon showed

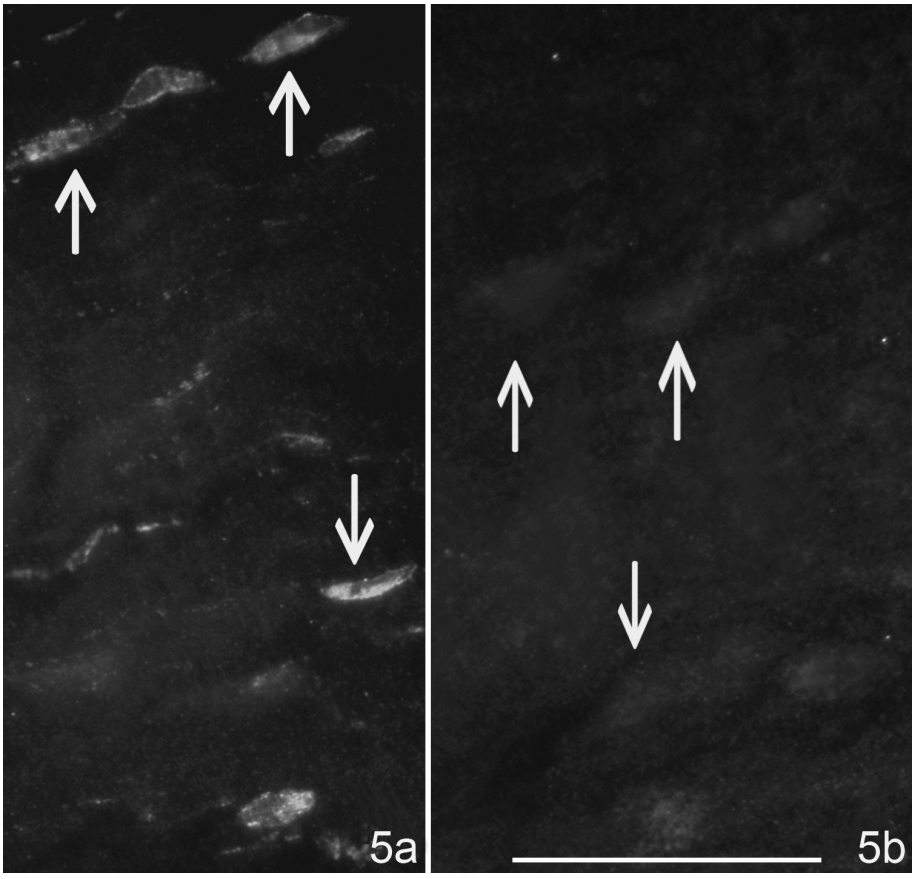

Fig. 5. Immunostaining of plantaris tendon tissue for demonstration of ChAT **(a)** and ChAT after preabsorption with ChAT antigen **(b)**. Achilles tendinosis case. Parallel sections in the series. The area shown in **(b)** is similar to the area that is shown in **(a)**. The tenocytes show immunoreactions in **(a)** but not in **(b)**. Arrows at some of the occurring tenocytes. Bar: 10 μm.

ChAT immunoreaction all through the specimen (Fig. 6c). However, occasional tenocytes only showed unspecific background reactions. The tenocytes did not show CD68 immunoreactions and did not exhibit immunoreactions after processing with the T cell/neutrophil marker antibody (not shown).

Loose peritendinous connective tissue

As described above, loose peritendinous connective tissue was encountered in some of the specimens of plantaris tendons of cases with Achilles tendinosis, and this tissue was also present in the control plantaris specimen. The cells that were located in this tissue did collectively show a marked ChAT immunoreaction (Fig. 6a,b). Cellular reactions for fibroblast marker (Fig. 6a, inset) were also very frequently detected for cells located in this tissue. Furthermore, some of the cells in this location showed CD68 immunoreactions. This was the case in tendinosis specimens as well as for the control plantaris specimen (Fig. 7). The cells in the peritendinous connective tissue never showed reactions when the sections were incubated with the T cell/neutrophil antibody (not shown).

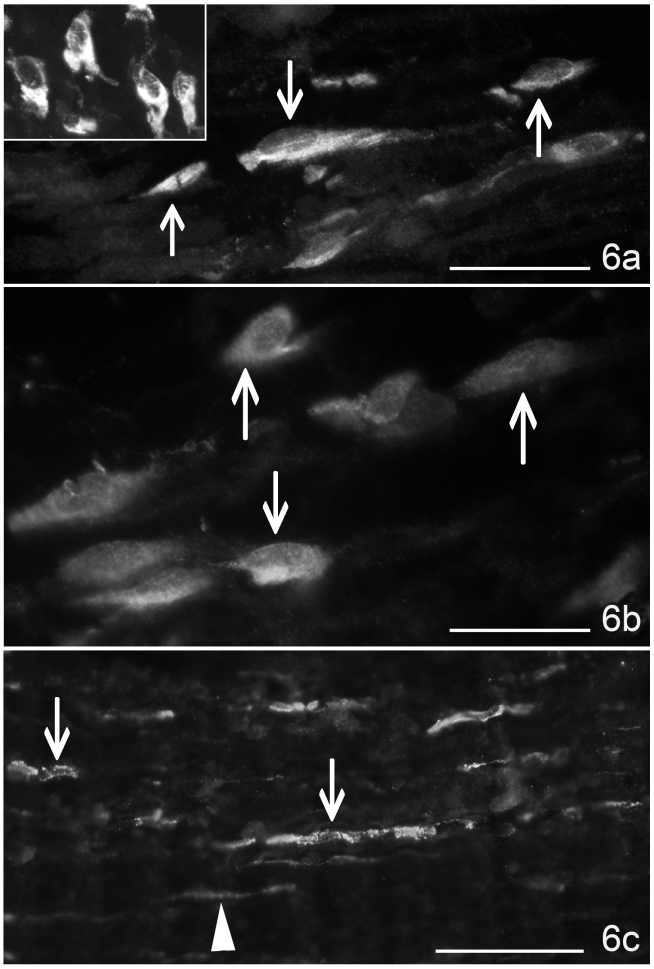

Fig. 6. Occurrence of ChAT immunoreactions in cells located in the loose peritendinous connective tissue between the plantaris and Achilles tendons is shown **(a, b)**. Arrows at some of the cells. The cells in this area also show immunoreactions for fibroblast marker (inset **a**). In **(c)**, a part of a normal plantaris tendon is shown. Distinct ChAT immunoreactions are seen in the slender tenocytes (arrows). However, there are also tenocytes only exhibiting background reactions (arrowhead). Bars: 10 μm.

Discussion

This study shows that the excised plantaris tendons from patients with midportion Achilles tendinosis clearly show morphological features that are similar to those previously demonstrated for the tendinosis Achilles tendon (Astrom and Rausing, 1995; Movin et al., 1997; Khan et al., 1999; Maffulli et al., 2004; Shalabi, 2004; Bjur et al., 2005, 2008) as well as the tendinosis patellar tendon (Cook et al., 2004; Danielson et al., 2006). These include a very frequent occurrence of tenocytes with abnormal and irregular appearance and a lining-up of widened/rounded tenocytes. Overall, a large number of tenocytes was seen in the tendon tissue. Such features were, as expected, seen in Achilles tendons of the patient

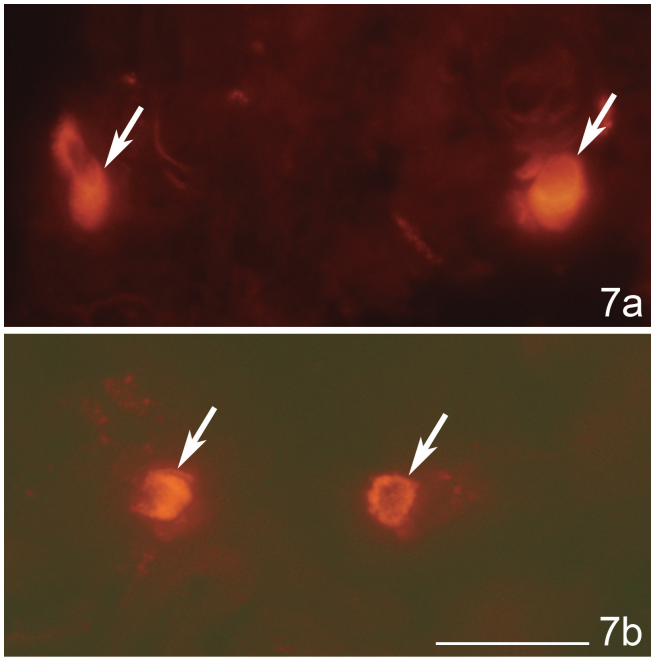

Fig. 7. Sections showing parts of loose peritendinous connective tissue located just outside plantaris tendons; tendon of Achilles tendinosis patient **(a)** and control plantaris tendon **(b).** There are cells that show CD68 immunoreactions (arrows). Bar: 20 μm.

group examined here.

To the best of our knowledge, this is the first study where tendinosis features have been described for the plantaris tendon. These were hereby shown to co-exist with tendinosis changes in the nearby located Achilles midportion in cases with typical Achilles tendinosis. All patients thus had midportion Achilles tendinosis, verified clinically and by ultrasound. The tendinosis changes in the plantaris tendon were found in the entire tendons examined and not only in the part involved in invagination into the Achilles tendon.

The findings that plantaris tendons exhibit an abnormal morphology show that the plantaris tendon, similarly as the Achilles tendon itself, is affected in cases with midportion Achilles tendinosis when the plantaris is closely associated with the Achilles tendon. This is a situation that is often encountered (c.f. above). The plantaris tendon thus shows tendinosis-like morphological features and may be a co-factor, besides the Achilles tendon itself, that needs to be considered in patients with medial Achilles tendon pain. I.e. there appears to be an involvement of the plantaris tendon in this situation. In accordance with this suggestion, recent findings show that excision of the plantaris tendon in patients with medial pain from midportion Achilles tendinosis was of significant clinical value (Alfredson, 2011a; van Sterkenburg et al., 2011). Of interest, there are also recent findings that the plantaris is a stiffer and stronger tendon than the Achilles tendon (Lintz et al., 2011). With regard to the tendons examined in this study, the plantaris tendons were always located close to, and sometimes even invaginated into, the medial Achilles tendon. Whether one of the two tendons provokes the other to start the process of tendinosis is not known, but the "compression theory" that is being discussed for insertional tendinopathy/tendinosis (Cook and Purdam, 2009) might possibly be relevant for midportion Achilles tendinopathy/tendinosis. When relating to a possible "compression theory", a very close apposition between the plantaris and Achilles tendons in Achilles tendinosis conditions i.e. when the plantaris tendon is located very close to or coalesced with the Achilles tendon might theoretically be associated with compressive forces between the two tendons.

The tenocytes in the plantaris tendons in cases with mid-portion Achilles tendinosis showed marked ChAT immunoreactions. Also, tenocytes in the control plantaris tendon exhibited such immunoreactions. ChAT immunoreactions are typically seen in tenocytes in Achilles (Bjur et al., 2008) and patellar (Danielson et al., 2006) tendinosis tendons but are weakly detected in normal and pain-free Achilles (Bjur et al., 2008) and patellar (Danielson et al., 2006) tendons. The findings of ChAT immunolabelling for tenocytes in the plantaris tendons suggest that there is a local production of acetylcholine (ACh) within the tendon tissue of not only the Achilles and patellar tendons, which previous observations favour (Bjur et al., 2008, Danielson et al., 2008), but also in the plantaris tendon.

Cells, lying scattered or in groups, were frequently found in the loose peritendinous connective tissue located between the plantaris and Achilles tendons. The cells were demarcated by a fibroblast marker and showed a marked ChAT immunoreaction. This finding suggests that there is a local production of ACh, not only in the tenocytes of the plantaris tendon, but also in cells in the loose peritendinous connective tissue. Similarly, it has been found that fibroblasts, as well as mononuclear-like cells, in the synovial tissue of patients with advanced rheumatoid arthritis and osteoarthritis display ChAT immunoreactions (Grimsholm et al., 2008). It has also been observed in other situations that ACh can be produced in non-neuronal cells such as cultured fibroblasts (Lips et al., 2003), skin cells (Kurzen et al., 2007) and inflammatory cells (Kawashima and Fujii, 2003). These findings have led to the recognition of a "non-neuronal cholinergic system" in various parts of the body (Wessler and Kirkpatrick, 2008)

The fact that cells located both within the tendon tissue of the Achilles (Bjur et al., 2008) and plantaris (present study) tendons, as well as cells in the loose peritendinous connective tissue between the two tendons (present study), show ChAT immunoreactions, which suggest that ACh-mediated effects may be of functional importance in all three regions (Fig. 8). These effects may be related to proliferative and collagen-modulating effects. Stimulation of ACh receptors is thus shown to have effects on collagen production (Jacobi et al., 2002, Sekhon et al., 2002) and ACh has effects on hepatic stellate cells in the form of both effects on proliferation

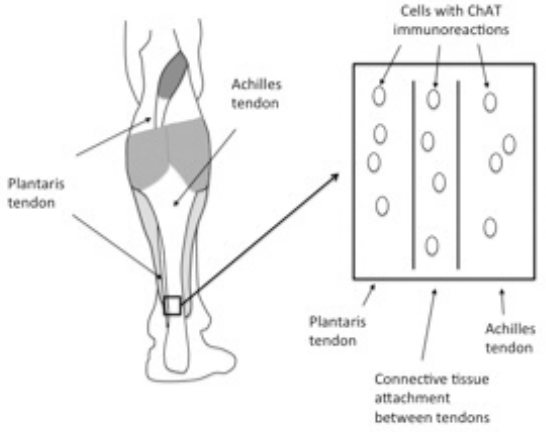

Fig. 8. Schematic drawing of the Achilles/plantaris tendon region (left). Principle schematic drawing illustratring the occurrence of ChAT immunoreactions in the tenocytes of the Achilles and plantaris tendons and in local cells (fibroblasts) located in the loose connective tissue inbetween the two tendons (right).

and collagen expression (Oben et al., 2003). As described above, it is well-known that hypercellularity is a characteristic feature of tendinosis tendons, and effects on the collagen structure, in the form of a discontinuity of the collagen, occurs in tendinosis (Khan et al., 1999).

Some cells in the loose peritendinous connective tissue of specimens of tendinosis tendons showed CD68 immunoreactions. This shows that macrophages are present here. It appears as if this is the situation for normal tendons as well, as CD68 immunoreactive cells were also encountered in this tissue in the control tendon specimen.

In conclusion, the present study shows that plantaris tendons exhibit similar histopathological changes as Achilles tendons in midportion Achilles tendinosis in cases when the plantaris tendon is closely associated with the Achilles tendon. The plantaris tendons thus also exhibit tendinosis-like features in this situation. These findings show that the plantaris tendon is coupled to the Achilles tendon in the processes that occur in midportion Achilles tendinosis. The tenocytes of the Achilles tendon (Bjur et al., 2008), as well as the plantaris tendons and local cells in the loose peritendinous connective tissue located inbetween the plantaris and Achilles tendons, furthermore show a marked ChAT immunoreaction. The functional significance of the apparent ACh production must await further studies.

Acknowledgements. The authors acknowledge Ms Ulla Hedlund for excellent technical services and PhD Lena Carlsson for help with photo montages. Financial support was obtained from the Faculty of Medicine at Umeå University and the Swedish National Centre for Research in Sports.

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Accepted November 7, 2012