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Further proof of the existence of a non-neuronal cholinergic system in the human Achilles tendon: Presence of the AChRα7 receptor in tendon cells and cells in the peritendinous tissue

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ABSTRACT

Human tendon cells have the capacity for acetylcholine (ACh) production. It is not known if the tendon cells also have the potential for ACh breakdown, nor if they show expression of the nicotinic acetylcholine receptor AChRα7 (α7nAChR). Therefore, tendon tissue specimens from patients with midportion Achilles tendinopathy/tendinosis and from normal midportion Achilles tendons were examined. Reaction for the degradative enzyme acetylcholinesterase (AChE) was found in some tenocytes in only a few tendinopathy tendons, and was never found in those of control tendons. Tenocytes displayed more regularly α7nAChR immunoreactivity. However, there was a marked heterogeneity in the degree of this reaction within and between the specimens. α7nAChR immunoreactivity was especially pronounced for tenocytes showing an oval/widened appearance. There was a tendency that the magnitude of α7nAChR immunoreactivity was higher in tendinopathy tendons as compared to control tendons. A stronger α7nAChR immunoreactivity than seen for tenocytes was observed for the cells in the peritendinous tissue. It is likely that the α7nAChR may be an important part of an auto-and paracrine loop of non-neuronal ACh that is released from the tendon cells. The effects may be related to proliferative and blood vessel regulatory functions as well as features related to collagen deposition. ACh can furthermore be of importance in leading to anti-inflammatory effects in the peritendinous tissue, a tissue nowadays considered to be of great relevance for the tendinopathy process. Overall, the findings show that tendon tissue, a tissue known to be devoid of cholinergic innervation, is a tissue in which there is a marked non-neuronal cholinergic system.

1. Introduction

It has become established that tendon tissue is among the tissues where there is a non-neuronal cholinergic system. There is thus expression of the acetylcholine (ACh)-synthesizing enzyme choline acetyltransferase (ChAT) in the tenocytes of the patellar [1], Achilles [2] and plantaris [3] tendons. There is also expression of the ACh-transporting enzyme vesicular ACh transporter (VAChT) [2]. The expressions were especially marked in tendons of tendinopathic/tendinosis tendons. The tenocytes have furthermore been shown to exhibit muscarinic M2 immunoreactions [1,2]. It was concluded that the cholinergic system can have an unexpected function in tendon tissue [4].

There are, however, aspects that are unclear concerning the cholinergic system for tendon tissue of mammalian species. That includes a lack of information as to whether there is also the other central aspect of ACh metabolism, namely ACh degradation, in the tenocytes. It is also unknown if there is a presence of the nicotinic acetylcholine receptor AChRα7 (α7nAChR) in tenocytes. This receptor has been shown to be very important in other tissues, being involved in inflammatory and remodulating events, especially having anti-inflammatory effects [5,6]. Concerning tendon tissue, it is known that the α7nAChR is found in tendon fibroblasts from chick embryos [7].

In the present study, tendon tissue from patients with chronic painful midportion Achilles tendinopathy (tendinosis) and from individuals with pain-free normal Achilles tendons were evaluated concerning histochemical expressions of the ACh-degrading enzyme acetylcholinesterase (AChE) as well immunohistochemical expression of the AChRα7 receptor. Midportion tendinopathy is the most frequent tendinopathy/tendinosis affecting the Achilles tendon.

2. Materials and methods

2.1. Patients

Achilles tendon tissue samples mainly containing inner part of the tendon (tendon tissue proper) from in total 33 individuals, 16 males

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and 17 females, were investigated. The individuals corresponded to two groups (see below). Achilles tendon samples conforming to the outer part of the tendon (peritendinous tissue) from 4 individuals were also investigated (females; mean age 52 years).

One of the groups for which tendon tissue proper was evaluated conformed to 26 individuals, 13 males and 13 females (mean age of males: 49 years; mean age of females: 51 years) who had had a long duration of pain symptoms (range 12–36 months) from the midportion of the Achilles tendon. They had experienced activity related pain from a tender thickening in the Achilles midportion. Diagnosis was confirmed with ultrasonography, showing a thickening of the Achilles midportion including structural irregularities and hypo-echoic regions. All these patients were surgically treated, whereby tendon tissue samples were taken from the tendinopathy affected Achilles midportion. They are further on referred to as tendinopathy patients.

Another group for which tendon tissue proper was evaluated conformed to 7 individuals who were classified as representing a normal group (3 males — mean age 40 years; 4 females — mean age 40 years). They were pain-free and had normal tendons as seen by clinical examination and frequently also as verified by ultrasound examination. Also for these individuals, biopsies were taken from the Achilles midportion.

Individuals from whom peritendinous tissue was examined conformed to patients who displayed the same types of features concerning pain symptoms and ultrasonography characteristics as the group of 26 individuals referred to above. Thus, these also correspond to tendinopathy patients.

All patients and normal controls were otherwise healthy, on no medication and non-smokers.

The study protocol was approved by the Ethics Committee at the Faculty of Medicine and Odontology, Umeå University, and the Regional Ethical Review Board in Umeå (04-157 M). The procedures were conducted according to the principles expressed in the Declaration of Helsinki.

2.2. Sampling and fixation

A surgical procedure was performed under which strict sterile conditions were kept. The surgery was performed in local anaestesia, as previously described [2]. Specimens of tendon tissue proper or peritendinous tissue were taken and directly transported to the laboratory. They were either processed in an unfixed way or were chemically fixed. Unfixed specimens were directly mounted in OCT embedding medium (Miles Laboratories, Naperville, IL) and frozen at −80 °C (using propane chilled with liquid nitrogen). The procedures used for fixation included fixation via immersion overnight at 4 °C in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. These specimens were thereafter thoroughly washed in Tyrode's solution, containing 10% sucrose, and mounted and frozen as were the specimens described above. Sectioning, according to well established procedures in the laboratory, was performed (cf. e.g. [1–3]).

2.3. Principles for processing of the specimens for histochemistry and immunohistochemistry

As described above, some specimens were processed in a chemically unfixed way whereas others were used after chemical fixation. As the staining for demonstration of AChE activity histochemically requires that the specimens are processed chemically unfixed, these types of specimens were used for the AChE staining. α7nAChR demonstration does, on the other hand, require the use of fixed tissue, why fixed specimens were utilized for α7nAChR immunolabelling. Due to this fact, specimens conforming to tendon tissue proper from 17 individuals (12 tendinopathy/tendinosis patients and 5 controls) could be stained for AChE histochemistry and specimens from 20 individuals (14 tendinopathy/tendinosis patients and 6 controls) could be applied for α7nAChR immunolabelling. The 4 specimens containing peritendinous tissue were used for α7nAChR immunostaining.

2.4. Histochemical demonstration of AChE activity

Specimens processed in a chemically unfixed way were sectioned using a cryostat, the sections being cut at a thickness of 8–10 μm. Mounting was performed on slides pre-coated with chrom-alun gelatine, whereafter they were stained for the demonstration of AChE activity. The sections were postfixed in formol-sucrose fixative for 1 h at 4 °C, and treated with tetraisopropyl-pyrophosphoramide (iso-OMPA) ($(10^{-4}$ M)) as an inhibitor of non-specific cholinesterase in a 30 min pre-incubation. The following procedures conform to those previously described [8].

2.5. Immunohistochemical procedures for demonstration of α7nAChR

Series of 8–10 μm thick sections were cut using a cryostat, whereupon they were mounted in Vectashield hard set microscopy mounting medium (Dakopatts, Denmark). Immunohistochemical procedures followed, which in principle were as previously described [2,3]. Treatment with KMnO4 was applied in order to enhance specific immunoreactions [9,10]. Incubation of the sections was performed for 20 min in a 1% solution of Triton X-100 (Kebo lab, Stockholm) in 0.01 M phosphate buffer saline (PBS), pH 7.2, containing 0.1% sodium azide as preservative. Thereafter followed rinsing in PBS three times, 5 min each time, after which the sections were incubated with 5% normal donkey serum in PBS. Then followed incubation with the primary antibody, diluted in PBS, in a humid environment. This incubation was performed for 60 min at 37 °C. After this, the sections were washed three times for 5 min in PBS, whereafter a new incubation in normal donkey serum followed. Then the sections were incubated with secondary antibody. This proceeded for 30 min at 37 °C. As secondary antibody, a FITC-conjugated donkey anti-goat IgG (Jackson Immunoresearch, West Grove, PA), diluted 1:100, was used. The sections were thereafter washed in PBS and then mounted in Vectashield Mounting Medium (H-1000) (Vector Laboratories, Burlingame, CA, USA). A Zeiss Axioscope 2 plus microscope equipped with an Olympus DP70 digital camera was used for examination.

The primary α7nAChR antibody was an affinity purified goat polyclonal antibody raised against a peptide mapping at the C-terminus of α7nAChR of human origin (Santa Cruz Biotechnology; sc-1447, dilution used 1:100). Control stainings conformed to stainings for which the antibody had been preabsorbed with corresponding antigen (sc-1447P; Santa Cruz), at a concentration of 50 μg/ml, and to stainings when the specific antiserum was exchanged with buffer. The usefulness of the antibody has also previously been evaluated via preabsorptions in studies on human synovial tissue [10]. The usefulness of the currently used α7nAChR antibody has also been shown in the sense that it demarcates the same types of cells that are stained in the human synovial tissue via the use of other types of α7nAChR antibodies used by other research groups [11,12].

2.5.1. Scoring of immunoreactivity for tenocytes

A scoring was made concerning the degree of α7nAChR for tenocytes. The scoring was made in the following way: 3 + = marked immunoreaction in most parts of the sections, 2 + = moderate reactions in most parts of the specimens, 1 + = general weak reactions, and 0 = no specific reactions at all.

2.6. Htx eosin staining

Sections in parallel to those that were processed for demonstration of AChE activity or for α7nAChR immunoreactivity were stained for haematoxylin–eosin in order to demonstrate general tissue morphology.