

***In situ* hybridization studies favouring the occurrence of a local production of BDNF in the human Achilles tendon**

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Summary. Brain derived neurotrophic factor (BDNF) is a multipotent neurotrophin known for its growth-influencing and apoptosis-modulating functions, as well as for its function to interact with neurotransmitters/neuromodulators. BDNF is reported to be mainly produced in the brain. BDNF can be absorbed into peripheral tissue from the blood stream. Expression of this neurotrophin at the protein level, as well as of the neurotrophin receptor p75, has been previously shown for the principal cells (tenocytes) of the Achilles tendon. However, there is no proof at the mRNA level that BDNF is produced by the tenocytes. As the Achilles tendon tenocytes show “neuronal-like” characteristics, in the form of expressions favouring synthesis of several neuromodulators/neurotransmitters, and as BDNF especially is produced in neurons, it is of interest to confirm this. In the present study, therefore, *in situ* hybridization for demonstration of BDNF mRNA was performed on biopsies from Achilles tendons of patients with tendinosis and pain-free non-tendinosis individuals. The results showed that the tenocytes of both groups exhibited BDNF mRNA reactions. These observations indeed favour the idea that BDNF is produced by tenocytes in the human Achilles tendon, why Achilles tendon tissue is a tissue in which BDNF can be locally produced. BDNF can have modulatory functions for the tenocytes, including apoptosis-modifying effects via actions on the p75 receptor and interactive effects with neurotransmitters/neuromodulators produced in these cells. This possibility should be further studied for Achilles tendon tissue.

Key words: Achilles tendon, BDNF, mRNA, *In situ* hybridization, Tendinopathy

Introduction

Neurotrophins are mainly produced in the brain. These substances are important for the maintenance, survival, protection and differentiation of neurons (Linnarsson et al., 2000) but are also important factors in inflammatory and growth processes (Levi-Montalcini et al., 1990). Neurotrophins are mainly produced in neurons and inflammatory cells (Nockher and Renz, 2005). One of these neurotrophins is brain derived neurotrophic factor (BDNF). Approximately 75% of the circulating BDNF is produced in the brain, but BDNF easily passes the blood-brain barrier (Rasmussen et al., 2009). BDNF is known to play neuromodulatory roles (Lin et al., 2011), including a role in chronic inflammatory disease (Rössing et al., 2011), and to have a critical role in the bidirectional signaling mechanisms between immune cells and neurosensory network structures (Scuri et al., 2010). BDNF shows interactive effects with neurotransmitters and neuromodulators (Paredes et al., 2007; Meuchel et al., 2011) and acts via the high affinity tyrosine kinase receptor TrkB and the low affinity neurotrophin receptor p75 (Martin-Zanca et al., 1989; Chapman and Kuntz, 1995).

We have previously found evidence suggesting that the tendon tissue of the human Achilles tendon is a tissue in which BDNF can be locally produced. BDNF immunoreactivity was shown for the principal cells (the tenocytes) of both the normal Achilles tendon and the Achilles tendon in patients with chronic Achilles tendon pain (Achilles tendinosis) (Bagge et al., 2009). However, there were variations in the degree of tenocyte immunoreactions within the specimens. The finding of BDNF immunoreactivity in the tenocytes is very unexpected, as the tenocytes by definition are non-neuronal and non-inflammatory cells, and because there is no inflammatory component in Achilles tendinosis (Alfredson et al., 2002; Riley, 2004).

There is no confirmatory information concerning

BDNF expression for tenocytes at the mRNA level. Thus, although the immunohistochemical results suggest that BDNF is produced by tenocytes, the results do not prove this. BDNF is present in the blood and the circulating BDNF can actually be absorbed into peripheral tissues (Knaepen et al., 2010). Although it seems unlikely, it can therefore not be completely excluded that observations of BDNF at the protein level in tenocytes could be related to an uptake from circulating BDNF.

In view of what is described above, it is important to get confirmatory information concerning BDNF at the mRNA level for Achilles tendon tissue; to find out if there really can be a local BDNF production in Achilles tendon tissue. An interesting aspect is that the tenocytes in the human Achilles tendon actually show features, which favour the production of neurotransmitters and neuromodulators (Bjur et al., 2008a,b; Scott et al., 2008). Although they are not neurons, they can, to some extent be looked upon as “neuronal-like”. The tenocytes form a network in the tendon tissue, the signaling within which is largely unknown. Identification of biochemical markers/signal substances for the tenocytes can give a further understanding for this signaling (Riley, 2004). One substance that could be of functional significance is BDNF.

In this study we therefore analysed biopsies of Achilles tendons for BDNF mRNA expression. Both normal tendons and tendons of patients suffering from tendinosis were examined. The aim was to clarify if the tendon cells of both groups indeed show expression for BDNF at the mRNA level, giving further evidence of a possibility that the cells produce this neurotrophin. We hypothesised that the tenocytes show such an expression.

Materials and methods

Individuals

Achilles tendon biopsies were randomly selected from a larger collection of biopsies from 1) patients with long lasting (>3 months) painful mid-portion Achilles tendinopathy who had not responded to conservative treatment (i.e. eccentric training) and were therefore surgically operated at the Sports Medicine Clinic in Umeå, Sweden [one male, age 29, and one female age 52], and 2) from randomly selected control individuals with pain free Achilles tendons with no structural changes [two females both at the age of 47]. Tendon morphology was verified by ultrasonography with Colour Doppler for both groups. The patients with tendinopathy but not the controls, showed abnormal tendon structure. When diagnosed by abnormal tendon tissue structure by ultrasonography or biopsy, the condition can be referred to as Achilles tendinosis (Alfredson, 2005). The term “tendinosis” is further on used in this study.

Specimens from a large collection of Achilles tendon biopsies present in the laboratory (c.f above) had

all previously been investigated concerning BDNF immunoreactivity patterns, and it was hereby noted that the tenocytes in these showed varying degrees of BDNF immunolabelling (Bagge et al., 2009). That included specimens from the tendons here examined. For comparison, specimens from these four tendons were re-sectioned and re-examined with respect to BDNF immunolabelling in the present study. In addition, a specimen containing colonic tissue from a patient operated due to ulcerative colitis (UC) was used as reference specimen concerning the in situ hybridization.

Sampling

Biopsies were taken during surgical treatment for the tendinosis group. Under local anaesthesia (4-5 ml pilokainhydrochloride, 10 mg/ml, Södertälje, Sweden), tendon tissue (macroscopically abnormal) was taken from the ventral part of the Achilles tendon through a longitudinal incision lateral to the tendon mid-portion. Tissue samples were taken from different depths of the tendons and were approximately 2 mm wide and 1-5 mm long. From the non-tendinosis tendons, samples of the same size were collected from the dorsal part of the tendon using a longitudinal plain incision under local anaesthesia (4-5 ml pilokainhydrochloride, 10 mg/ml, Södertälje, Sweden)

Fixation and sectioning

Immediately after the tendon samples were taken, they were transported to the laboratory and fixed overnight at 4°C in a solution of 4% formaldehyde in 0.1 M phosphate buffer, pH 7.0. The samples 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. The control tissue (colonic tissue) was processed in a similar way. Sections of 10 µm thickness from all samples were cut using a cryostat (Leica Microsystem CM 3000, Heidelberg, Germany) and processed for in situ hybridization, and other sections of 7-8 µm thickness were stained with haematoxylin-eosin, in order to explore tissue morphology, or processed for immunohistochemistry.

In situ hybridization (ISH)

Digoxigenin (DIG)-hyperlabelled oligonucleotide probe (ssDNA) for detection of human BDNF (code: GD 1259-OP) mRNA was used (Gene Detect, New Zealand) with a sequence of BDNF: AGTTCCAGTGCCTTTTG TCTATGCCCCTGCAGCCTTCCTTGTTGTAACCC.

ISH was performed according to an established protocol (Panoskaltis-Mortari and Bucy, 1995), using an alkaline phosphatase (AP)-labelled anti-DIG antibody for detection, with a few modifications (Danielson et al., 2007; Andersson et al., 2008).

BDNF mRNA in the Achilles tendon

Series of 10 μm thick cryosections were cut using a cryostat (with a knife washed in 70% EtOH in diethyl pyrocarbonate [DEPC]- H_2O) and mounted on Super Frost Plus slides (nr. 041300, Menzel-Gläser, Braunschweig, Germany). The sections were air-dried at room temperature (RT) for 30 min, and then fixed in filter-sterilised 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4) for 60 min at RT. The slides were then washed twice with 2 x saline sodium citrate (SSC) for 10 min (20 x SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0 [20°C], autoclaved, 2 x SSC: 10 x dilution of 20 x SSC in DEPC- H_2O). The sections were incubated in 0.2 M HCl for 8 min at RT to inhibit endogenous AP activity, following which the sections

were acetylated by incubation of the slides for 15 min at RT in a mixture of 195 ml DEPC- H_2O , 2.7 ml diethanolamide, 0.355 ml HCl, and 0.5 ml acetic anhydride (the acetic anhydride being added after the slides had been placed in the slide holder). Slides were then again rinsed in 2 x SSC. An aliquot (50 ng) of the ssDNA probe was added to 15 μl of hybridization solution in a 1.5 ml microfuge tube, denatured for 5 min in 80°C and then put on ice. The hybridization solution was as follows: 500 μl formamide, 200 μl 20 x SSC, 50 μl of 20x Denhardt's solution, 50 μl herring sperm DNA [10 mg/ml] heat-denatured, 25 μl bakers yeast RNA [10 mg/ml], 175 μl dextran sulphate [50%]; total volume: 1.0 ml. The probe-containing hybridization

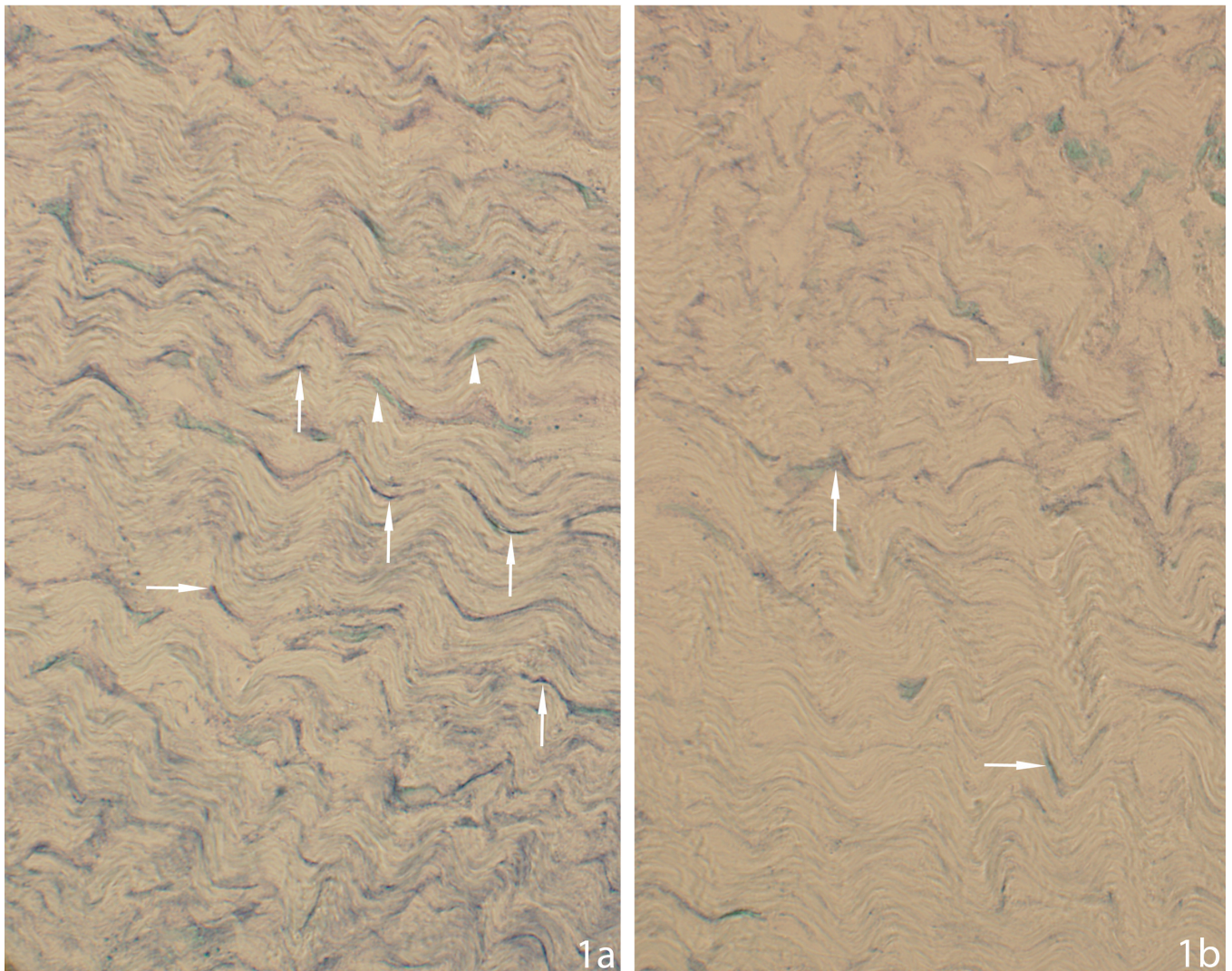


Fig. 1. Parallel slides of sections from a tendinosis patient processed with antisense (a) and sense (b) probes for demonstration of BDNF mRNA. Strong mRNA reactions in the form of black granular reactions are detected in a large number of the tenocytes (white arrows) (a). Other tenocytes do not show these reactions (white arrowheads). Only weak reactions of background type are seen in the tenocytes in (b). Arrows in (b) point at tenocytes. x 200

BDNF mRNA in the Achilles tendon

solution was then applied to each section, the sections being covered with cover slips and sealed with nail polish. Incubation followed at 56°C overnight.

The slides were washed twice for 10 min at RT in 2 x SSC and for 5 min at RT in STE-buffer (STE-buffer: 500 mM NaCl, 20 mM Tris-HCl [pH 7.5], 1 mM EDTA). Subsequently, the slides were incubated in 100 μ l RNase A (40 mg/ml in STE) for 30 min at 37°C, following which they were washed for 20 min at 56°C in 2 x SSC, 50% formamide (25 ml 100% and 25 ml 2 x SSC buffer), diluted up to 50 ml with double-distilled H₂O, set into a 56°C bath, then for 2x5 min placed at RT in 1 x SSC, and for 2x5 min at RT in 0.5 x SSC. Then the slides were washed for 5 min in buffer 1 (100 mM Tris-HCl [pH 7.5] containing 150 mM NaCl) followed

by incubation of the sections in buffer 1 containing 4% normal horse serum (NHS) for 60 min at RT in a humid chamber. The sections were incubated in 100 μ l of the AP-labelled anti-DIG anti- body (diluted 1:500 in buffer 1 supplemented with 4% NHS) for 60 min at RT in a humid chamber. The slides were washed twice for 10 min in buffer 1, and twice for 5 min in buffer 2 (100 mM Tris-HCl [pH 9.5] containing 100 mM NaCl and 50 mM MgCl₂). The enzyme (AP) substrate solution (20 μ l NBT/BCIP in 1 ml buffer 2 with 10 μ l levamisole) was sterile filtered (22 μ m) and added to the sections, and the slides were incubated inverted in the dark at 4°C overnight. The colour reaction was stopped by placing the slides in buffer 3 (10 mM Tris- HCl [pH 8.0] containing 1 mM EDTA).

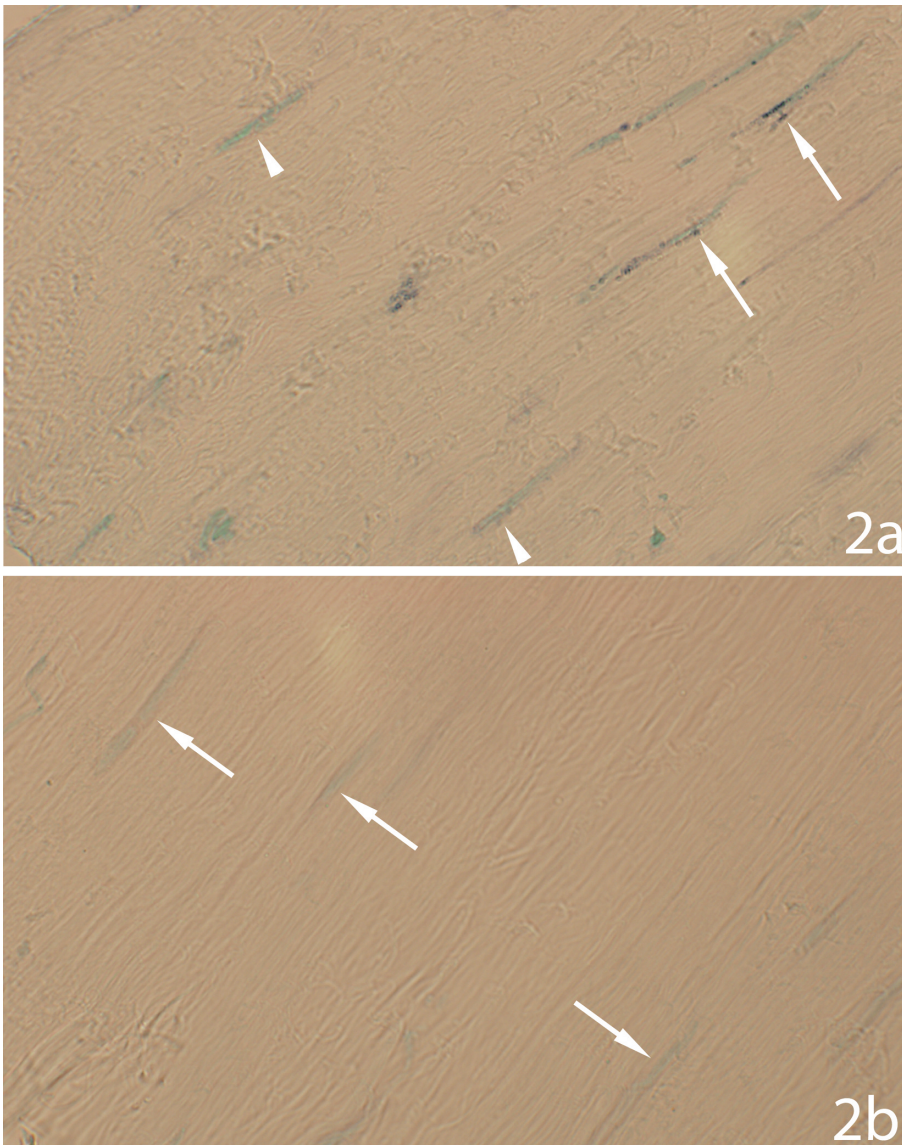


Fig. 2. Parallel slides of sections from a non-tendinitis individual, using antisense (a) and sense (b) probes, for demonstration of BDNF mRNA. Strong mRNA reactions, in the form of black granular reactions, are detected in parts of the tenocytes in (a) (arrows). Non-reactive tenocytes in (a) are marked with arrowheads. Weak reactions of a background type are seen when the sense probe was applied (b). Arrows in b point at tenocytes. x 200

BDNF mRNA in the Achilles tendon

Counter-staining of the slides in 0.5% methyl green followed by immersing the slides for 30 s in 75% ethanol, for 30 s in 95%-ethanol, for 4-5 s in 0.5% methyl green, and finally washing three times in 99.5% ethanol. The slides were mounted in Pertex microscopy mounting medium. The corresponding sense DIG-hyperlabelled ssDNA probe was used as negative control and a β -actin probe (GD5000-OP, Gene Detect, New Zealand) was used as a positive control. All sections were evaluated by two observers (J.B. and S.F.)

Immunohistochemistry

Immunohistochemical staining for demonstration of BDNF was performed. The procedures were as previously described (Bagge et al., 2009). The BDNF antibody used was obtained from Santa Cruz Biotechnology Inc (California, USA) and was raised in rabbit (sc 546, dilution 1:100).

Results

General morphology

Biopsies from pain-free non-tendinosis individuals' tendons contained well-organized, parallel fiber bundles of collagen, and a moderate number of slender and spindle-shaped tenocytes. Tendinosis tendons contained a large number of tenocytes which showed wavy and thickened appearances but the tendon tissue also partly contained normally appearing tenocytes (not shown). These observations are in accordance with previous findings for normal and tendinosis Achilles tendons (Bjur et al., 2005; Bagge et al., 2009).

In Situ Hybridization (ISH)

Tendinosis tendons

There was a clear difference concerning expression patterns after processing with antisense and sense probes. A large number of the tenocytes in biopsies from tendinosis tendons showed markedly black reactions after staining with the antisense probe. The reactions were of a granular type (Fig 1a). That was the case both for slender, normally-appearing tenocytes as well as for wavy and thickened tenocytes. Some tenocytes did not show these black granular reactions (Fig. 1a). In sections processed with the sense probe, no black granular reactions were seen (Fig. 1b). It can therefore be concluded that a large number of the tenocytes showed specific BDNF mRNA reactions.

Non-tendinosis tendons

Specific mRNA reactions were also noted for some of the tenocytes in the biopsies from non-tendinosis patients (Fig 2a,b). A similar difference between tenocyte reactions after processing with antisense and

sense probes, as was seen in tendinosis tendon biopsies, was thus observed for these control samples. The tenocytes did not exhibit black granular reactions but

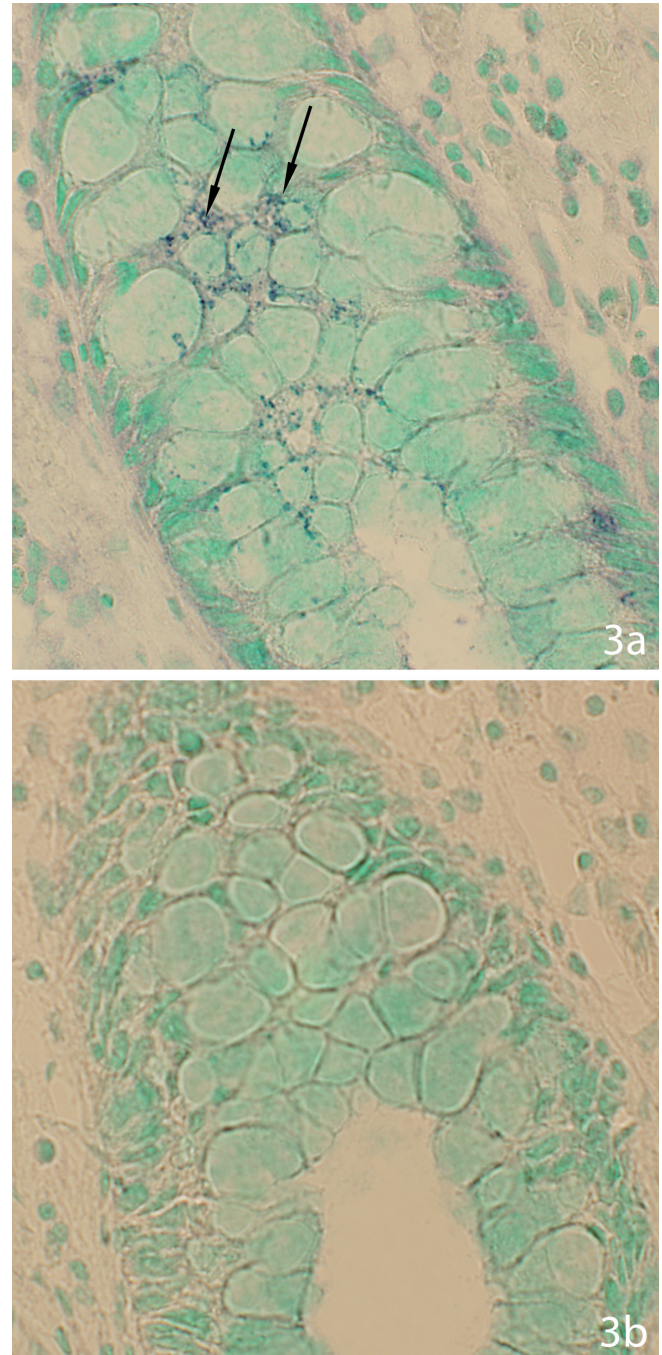


Fig. 3. Parallel slides of sections from a specimen of an ulcerative colitis patient, using antisense (a) and sense (b) probes, for demonstration of BDNF. Strong mRNA reactions in the form of granular reactions are seen in epithelial cells in (a). Arrows point at specific reactions in these cells. x 200

showed in principle only background reactions after processing with sense probe (Fig. 2b). The presence of black granular reactions was clearly obvious for the reactive tenocytes after staining with the antisense probe (Fig. 2a). Nevertheless, some of the tenocytes did not show BDNF mRNA reactions (Fig. 2a).

Control tissue

In colonic tissue from the ulcerative colitis patient, specific mRNA reactions were seen in epithelial cells. The reactions were also in this case seen in the form of black granular reactions (Fig. 3). Such reactions were not seen when the sense probe was applied (Fig. 3). It should be recalled that it has been previously shown that there is BDNF expression at the protein level in human intestinal epithelial cells (Hannestad et al., 1998; Johansson et al., 2007).

BDNF immunohistochemistry

It was noted that there was a variability in the degree of BDNF immunoreactivity in the tenocytes, i.e. a large number of the tenocytes showed BDNF immunoreactivity in both tendinosis (Fig. 4a) and non-

tendinosis (Fig. 4b) tendons. However, some tenocytes were not immunoreactive for BDNF (Fig. 4a,b).

Discussion

BDNF mRNA reactions were evident for a large number of the tenocytes in both tendinosis and healthy Achilles tendons. The results from this study thus add evidence to the theory that BDNF is produced in the tenocytes of the human Achilles tendon. The results of the present study therefore favour the finding of BDNF expression at the protein level for tenocytes (Bagge et al., 2009) is not related to uptake mechanisms from the blood, but instead a cellular production. However, it is obvious that BDNF immunolabelling and BDNF mRNA is not confined to all tenocytes in the Achilles tendon.

Functionally, it may be that BDNF is of importance for modulatory functions for the tenocytes. The suggestion that BDNF has own effects on tenocytes is supported by the fact that these cells show expression for the neurotrophin p75 receptor (Bagge et al., 2009), a receptor to which BDNF binds (Chapman and Kuntz, 1995). BDNF can thus have effects on the modulation of apoptosis events, as the p75 receptor mediates apoptotic

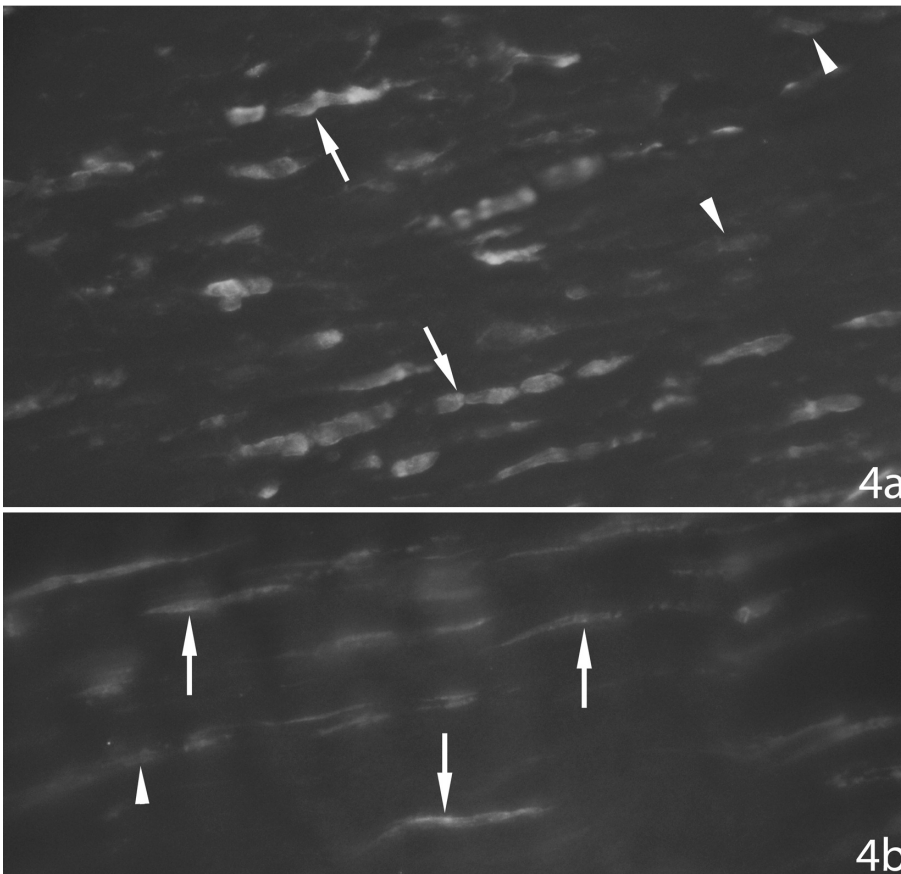


Fig. 4. Slides from specimens of tendinosis (a) and non-tendinosis (b) individuals stained for BDNF using immunohistochemistry. The tenocytes show a widened appearance and are frequently lined up in rows in the tendinosis tissue (a), whilst they show an elongated form in non-tendinosis tissue (b). Most of the tenocytes show specific immunoreactions in both a and b (arrows). Some of them are, however, not immunoreactive (arrowheads). x 200

modulatory functions (Kumar et al., 2006). It should here be remembered that apoptosis is a central event in tendinosis (Lian et al., 2007). It can also not be excluded that BDNF may have trophic, survival promoting and repair roles, like the situation in the nervous system (Linnarsson et al., 2000; De Santi et al., 2011), a protective role, as has been shown for the inflamed gut (Reinshagen et al., 2000) and a role on proliferation, as is described for multiple myeloma cells (Sun et al., 2012). The possible function of BDNF for tendon tissue may not only be restricted to tendon tissue of the Achilles tendon. We have thus noted the presence of BDNF immunoreactions for tenocytes in the human plantaris tendon as well (unpublished observations).

The findings that BDNF is expressed at both the mRNA and protein level for tenocytes are of interest concerning the fact that tenocytes of the human Achilles tendon show neuronal-like characteristics. Some of the cells in the tenocyte population thus show expressions at both these levels for enzymes related to acetylcholine (Bjur et al., 2008a) and catecholamine (Bjur et al., 2008b) production. They also show expression for enzymes related to glutamate transport (Scott et al., 2008), and mRNA reactions for substance P (Andersson et al., 2008). It should be remembered that BDNF is mainly expressed in neurons, that the tenocytes of the Achilles tendon form a communicating network, and that in principle there are no nerve fibres within tendon tissue proper (Bjur et al., 2005). It is therefore possible that BDNF and other nerve-related substances are confined to this network instead of being related to typical nerve elements within the tendon tissue.

It may be that BDNF has interactive effects with neurotransmitters and neuromodulators shown to exist for the tenocytes, thereby having an involvement in the signaling within the tenocyte network. It is known that BDNF can interact with glutamate and catecholamine (dopamine) in the rat hippocampus (Paredes et al., 2007), that there are interactions between BDNF and substance P in the regulation of airway smooth muscle of rats (Meuchel et al., 2011), and that BDNF can influence acetylcholine-effects via an attenuation of the alpha7 nicotinic acetylcholine receptor in hippocampal interneurons (Fernandes et al., 2008).

Another possibility is that the functions of BDNF in the tenocyte network can be related to interference with the effects of the cytokine tumour necrosis factor alpha (TNF-alpha). We have thus recently noted that the tenocytes of the human Achilles tendon also show expression for TNF-alpha and the TNF receptors TNFRI and TNFRII (Gaida et al., 2012). It is known that TNF-alpha, similarly to BDNF, can mediate the processes of apoptosis (Vanden Berghe et al., 2004), but TNF-alpha may eventually also have an involvement in recovery and repair processes (Warren et al., 2002). It has been previously shown that there are interactive effects between neurotrophins and TNF-alpha. TNF-alpha is, for example, demonstrated to regulate the secretion of BDNF in human monocytes (Schulte-Herbruggen et al.,

2005). It has also been shown that anti-TNF treatment leads to an attenuation of BDNF levels in the dorsal root ganglion and spinal cord in studies on spinal cord damage in rats (Onda et al., 2004) and a reduction in the BDNF levels in the blood in patients with rheumatoid arthritis (Grimsholm et al., 2008).

In conclusion, the present study gives further support to the theory that BDNF is produced in Achilles tendon tenocytes. Achilles tendon tissue is thus a tissue where BDNF can be produced in non-neuronal cells. Although BDNF is mainly a brain-derived substance and is related to inflammatory conditions, it should not be ignored that there may be an existence of a local BDNF production in a tissue without inflammation and where, in principle, there are no nerve fibers. In our case this is shown for Achilles tendon tissue. A limitation with the current study is that it does not clarify in what way BDNF is involved in normal tenocyte function and in the pathophysiology in tendinosis. This should be further studied. In forthcoming studies, the possible involvement of BDNF in trophic and modulatory functions and in interactive effects with other signal substances should be evaluated.

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