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# EXPRESSION OF CELL ADHESION MOLECULE L1 IN THE LONG HEAD OF BICEPS TENDON

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**Abstract –** Several studies have proposed that the nervous system participates in nociception and tendon healing process. The neural cell adhesion molecule L1 (L1-CAM), which has an important role in neural development and nociceptive pathways, has been described in the past in the skeletal muscles and tendino-muscular junction. The role of this protein in tendon pathology is unknown. Here, we show that L1-CAM is expressed in human tendons. Samples of the long head of the biceps tendon (LHB) from six patients undergoing shoulder surgery were studied. Both Western blot and immunofluorescence revealed a strong expression pattern of L1-CAM. These L1-CAM positive cells were also Tuj1 positive, suggesting a neuronal origin. To our knowledge this is the first unequivocal evidence of the presence of L1 CAM in human tendons suggesting that it may play a role in organization of extracellular matrix and tendon pain.

**Key words:** Tendon of long head of biceps, L1-CAM, shoulder pain, tendinosis.

## INTRODUCTION

The tendon of the long head of biceps (LHB) may be a major cause of shoulder pain in certain shoulder pathologies (8).

The nature of the neuronal innervation of LHB tendon was studied immunohistochemically in human cadaveric tissue using neurofilament, calcitonin gene-related peptide, substance P, and tyrosine hydroxylase antibodies. It was found that this tendon contains a large network of sensory and sympathetic nerve fibers, predominantly near its insertion. These findings suggest that the tendon of the LHB has the neural elements responsible for the pain sensation (2).

In general, chronic, degenerative tendon disorders have been described under the term tendinosis. Without clear understanding, tendinosis appears to be a degenerative process whereas the normal tolerance of the tendon to stress is exceeded, and its healing mechanisms are irreversibly impaired. (9). Tendinosis represents the ultimate consequence of multiple microtraumatic events that cause disruption of the tendon's internal construction, and disintegration of the cells and matrix, witch failed to mature into normal tissue. Although the role of inflammation is still controversial, it has been understood that tendinosis is primarily degenerative condition since histopahological studies shown rather an absence of inflammatory cells in or around the lesion. Research has recently presented evidence that the nervous system plays an important role regarding degeneration, inflammation and pain (9,14).

The cell adhesion molecule L1 (L1-CAM) belongs to the immunoglobulin superfamily and is known to be crucial for the development of the neural system including neurite outgrowth, axon fasciculation, myelination and regeneration in adult nervous system (11). The presence of numerous cell adhesion molecules, including L1-CAM, in the skeletal muscles and tendomuscular junction has been described in the past (5,16).

Interactions between L1-CAM and extracellular matrix components play a significant biological role since they lead to the activation of intracellular signalling cascades (10). Recent studies suggest that L1-CAM is involved in neuropathic pain behaviors by activating intracellular signaling cascades in nociceptive pathways (18). These findings prompted us to investigate L1-CAM expression

in human LHB tendon.

## MATERIALS AND METHODS

### *Patients and tissue collection*

The University Hospital of Crete Ethics Committee, Greece, approved human tissue collection. Six LHB tendon samples were collected from patients undergoing shoulder surgery where a long biceps tenotomy and tenodesis were indicated. The mean age was 71,3 years. There were 4 women and 2 men. One patient suffered from rotator cuff tear and underwent cuff repair and tenotomy of the LHB. Five patients underwent hemiarthroplasty for four-part fracture of the proximal humerus. The tendon tissue was snap frozen and stored at –80 $^{o}$ C until it was used. The tendons showed no major signs of degeneration on hematoxylin and eosin staining and viewing under normal light microscopy.

### *Western blot analysis*

For the Western blot analysis the human tendone and mouse brain tissues were lysed in an ice cold buffer containing 85 mM Tris, pH 7.5, 30mM NaCl, 1mM EDTA, 120mM glucose, 1%Triton X-100, 60mM octyl Q-D glucopyranoside and protease inhibitors cocktail diluted 1:1000 (Sigma-Aldrich). Homogenates were vigorously vortexed and then centrifuged for 30 min at 13000g at 4 $^{o}$C to recover the supernatant. The samples were boiled for five minutes in a SDS gel loading buffer solution containing 100 mM DTT. The proteins of the samples were separated according to their molecular weight using SDS polyacrylamide gel electrophoresis. After electrophoresis, proteins were transferred onto a nitrocellulose membrane at 310 mA for 1 hour. Membranes were blocked for 1 hour in 5% fat-free milk in PBS (phosphate buffered saline) + 0,1% Tween-20 (PBST). Incubation with primary antibody for L1 (rabbit polyclonal 1:5000, kind gift from F.G. Rathjen, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany) and actin (mouse monoclonal 1:4000, Chemicon) was performed overnight at 4 $^{o}$ C, in blocking solution. The next day the membranes were three times washed with PBST and incubated for 1 hour at room temperature with HRP-conjugated secontary antibodies (Boehringer Mannheim Biochemicals, 1:5000). After washing, the bands were detected by enhanced chemiluminescence (Chemicon ECL kit).

### *Cryosectioning and Immunocytochemistry*

Tendon samples were fixed overnight in a solution of 4% paraformaldehyde in PBS at 4$^{o}$C followed by immersion in 30% sucrose in PBS at 4 $^{o}$ C overnight. The next day, the tissue was embedded in 7.5 % gelatine and 15 % sucrose in 0.1M PBS, and immediately frozen in a dry ice-methyl-butane bath. 10-µm thick cryostat sections were mounted on super-frost glass slides and stored at -20 °C. The sections were washed with PBS + Triton X-100 0.1% and subsequently blocked with 10% FBS (Fetal bovine serum) in PBS + 0.1% Triton X-100 for 1 hour at room temperature. Then, the slides were incubated with the anti-L1 antibody ( rabbit polyclonal 1:500, kind gift from F.G. Rathjen, Max-Delbrück-Centrum für Molekulare Medizin, Berlin, Germany) and the anti-tubulin beta III (Tuj1, Sigma 1:4000) overnight at 4$^{o}$C. Slides were washed and incubated with the appropriate fluorescein- conjugated secondary antibodies (goat anti- rabbit Alexa 488 or 555, 1:800, Molecular Probes) for 2 hours at room temperature. Cell nuclei were visualized with To-Pro 3 (diluted 1:1000 in PBS) and slides were covered with mounting medium (mowiol) and observed under a confocal laser-scanning microscope.

## RESULTS

### *Western blot analysis in tendon tissues*

The expression of L1-CAM protein was confirmed by western blot analysis of the tendon of the long head of biceps (figure 1). As a positive control for L1 CAM expression, we used cortical tissue from adult mouse. We could detect one band at 210-220 kDa in human LHB, similarly to mouse cortex, as well as a lower band at 140 kDa in mouse tissue, detected at lower quantities in human LHB (figure 1).

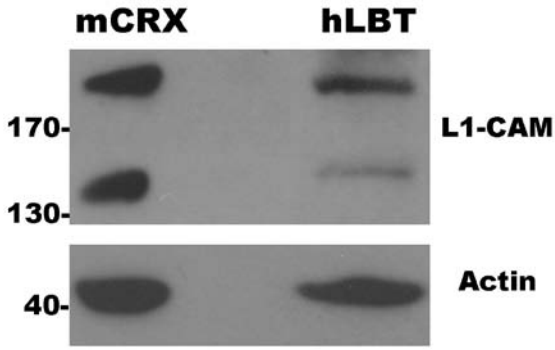


**Figure 1.** Western Blot analysis of mouse brain cortex and human LHB using antibodies against L1-CAM protein and actin as a loading control. A band of 210-220 kDa was detected for L1-CAM in both tissues, while a lower band was intense in murine tissue and more faint in human LHB.

### *Immonofluorescence labelling*

In longitudinal sections of the LHB tendon we observed cells strongly labelled for L1 CAM (green fluorescence). A double staining for L1 CAM (green fluorescence) and beta III tubulin, that is a marker of mature neurons (red fluorescence) revealed colocalization of the two proteins, suggesting that L1 CAM is expressed by neuronal cells found dispersed in LHB tendon (figure 2). To-pro 3 was used for nuclei labelling (blue fluorescence).

## DISCUSSION

In the present study we investigated the putative role of L1-CAM in the innervation of the LHB using Western blot analysis and immunofluorescence. To identify the cells that expressed L1-CAM we performed double immunostaining of L1-CAM with a protein

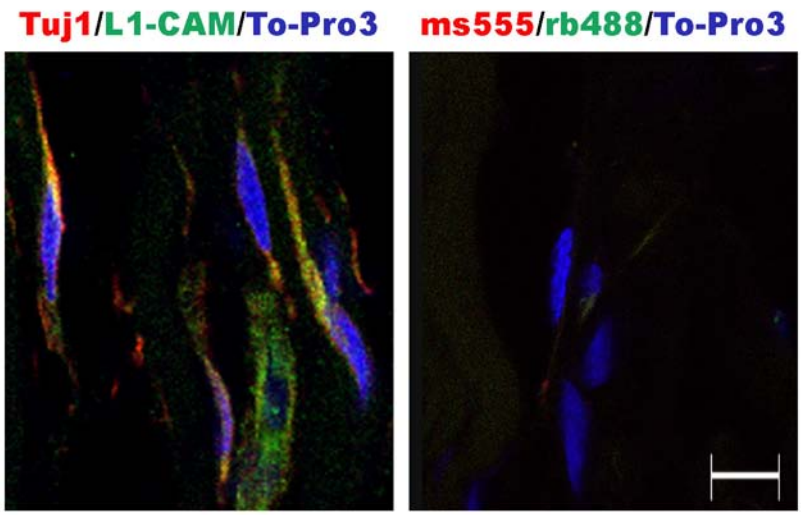


**Figure 2.** Immunostaining in longitudinal cryosections of human LHB stained with anti-L1-CAM antibody (green fluorescence) and Tuj1 antibody (red fluorescence) (left panel). The right panel depicts the experiment with fluorescent secondary antibodies alone. To-Pro3 for the nuclei staining is shown in blue. Scale: 150 μm.

marker of mature neuronal cells, beta III tubulin. Furthermore, we used To-pro3, which labels the cell nuclei. The observed images, suggested that the L1-CAM positive structures were mainly formed on the somata of neuronal cells found dispersed on human LHB.

The protein L1-CAM plays an extremely critical role in the development and the maintenance of the three-dimensional structure of the adult nervous system. This function is mainly achieved due to the connection with both intracellular and extracellular molecules. These extracellular interactions with proteoglycans have physiological significance to the organization of the extracellular matrix (11). Surprisingly little is known of the biology, the functional role and the developmental regulation of the neuronal cell adhesion molecules (CAMs) in the muscoloskeletal system. Also, the role of L1-CAM in tendon pathology is completely unknown. Nevertheless, there is evidence that neuronal CAMs influence the structural organization of muscoloskeletal tissues. It has been demonstrated that J1 protein binds with collagen fibers in skeletal muscles suggesting involvement in the building of the extracellular matrix (16).

In addition, more recent data demonstrate that other members of cell adhesion proteins family such as N-cadherin, and vinculin, contribute in the structural preservation of tendon tissue and in the cell signaling mechanisms (13).

Similar experiments examined the distribution of the neural cell adhesion molecule (NCAM) in rat skeletal muscle. NCAM was present on the surface of embryonic myotubes, but it was lost as development proceeded and was virtually absent from adult muscle. However, denervation of adult muscle results in the reappearance of NCAM (4). Additionally, Jamali et al showed that tenotomy leads to alterations in the expression of the NCAM in rabbit skeletal muscles (7).

Consequently, it seems that the expression of NCAM in muscle is regulated by the muscle's state of innervation, and NCAM may play a part in regulating muscle's susceptibility to innervation.

Given the documented function of L1-CAM in nociception as it is expressed in dorsal root ganglia (DRGs) and in the dorsal horn of spinal cord, it is perhaps not surprising that L1-CAM was present in the tendon tissue (6,15,17).

Prior work has established that peripheral nerve injury promotes post-transcriptional modifications in the expression of L1-CAM in (DRGs) and spinal cord. The authors observed that sciatic nerve transection altered the expression of L1-CAM protein but not the L1-CAM mRNA. In the same study, it was found that L1-CAM is co-localized with the protein GAP 43, which is a well-known marker of structural plasticity in the central nervous system. It is also known that both L1 CAM and GAP 43 contribute in neurite outgrowth and regeneration (12). The same study revealed that the protein L1- CAM might be involved in the activation of p38 MAPK and modulates synapses‘s

organization and plasticity. These data suggest that the modification of L1-CAM in nociceptive pathways may be an important pathomechanism of neuropathic pain (18).

The present study gives the first clear evidence of the presence of L1-CAM in human tendons. The LHB contains a population of neuronal cells, which are synthesizing this protein. Those cells are mature neurons, as it is indicated by Tuj1 protein expression. Tuj1 is expressed by neurons of the central and peripheral nervous system and has been used extensively as marker of neuronal differentiation. (1,3).

These results correlate with our earlier observation that a network of sensory and sympathetic fibers innervates LHB (2).

The expression of L1-CAM at the LHB suggests that it may be implicated in the regulation of the local state of innervation, and the nociception. The biological significance of L1-CAM expression in the LHB needs further investigation. The molecular mechanism underlying the possible involvement of this protein in the pathogenesis of LHB pain and how it might participate in the neurogenic inflammation model could be an important matter in tendinosis research.

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