Immunohistochemical Analysis of Neuronal Cell Markers of Filum Terminale in Tethered Cord Syndrome

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Abstract

Purpose: Filum Terminale (FT) is a fibrotic anatomical structure at the caudal end of the spinal cord. Structural changes in the FT can also be the cause of Tethered Cord Syndrome (TCS), which causes traction and stress in the spinal cord. The aim of this study is to show the neuronal cell markers Brain-Derived Neurotrophic Factor (BDNF), Glial Fibril Acidic Protein (GFAP), Nestin, Noggin in the FT samples of patients with TCS by comparing them with the samples obtained from normal cadavers.

Methods: In the study, FT groups were determined; as control FT group which is obtained from cadaver and TCS patients. GFAP, BDNF, Nestin and Noggin expressions in FT samples were shown by using immunohistochemistry technique.

Results: According to our study, we noted that neuronal cell markers GFAP, BDNF, Nestin and Noggin were expressed in the control group especially in the ependymal cells surrounding the central canal and continue to decrease in the TCS.

Conclusion: Identification of neuronal cell markers in the control filum terminale and their change in Tethered Cord Syndrome will provide important information in understanding the mechanisms underlying this disease.

Keywords: Tethered Cord Syndrome; BDNF; GFAP; Nestin; Noggin

Introduction

The Filum Terminale (FT) is a fibrous band extending from the conus medullaris to the upper of the coccyx. Disorders in development of the embryo that occurred in embryonal life or events that cause stretching of the spinal cord affect FT and disrupt its normal structure. The structural changes in Filum Terminale (FT), absence of elastic fibers in particular, cause Tethered Cord Syndrome (TCS) [1,2]. Histologic components of the control filum may include fibroconnective tissue, neuroglial tissue, peripheral nerve twigs and adipose tissue. Deterioration of collagen fibers in the FT, irregularity, deterioration of vascular structures, increase in adipose tissue and hyalinization can be seen in patients with TCS [3]. To date, many the studies on histologically structure of FT in patients with TCS have been performed, but there is inadequate to detect neuronal cell markers. The aim of this study is to examine the neuronal cell markers (BDNF, GFAP, Nestin, Noggin) and compare the expression patterns between TCS and normal FT samples.

Material and Methods

This study included 13 patients who were operated in the Neurosurgery Department of the Manisa Celal Bayar University School of Medicine from July 2005 to 2016. Ethics committee approval was obtained from the Manisa Celal Bayar University School of Medicine (No: 20478486-24). They were evaluated for epidemiologic, clinical data, radiologically, Somatosensory Evoked Potentials (SSEP) and urodynamical studies retrospectively. The ages of the patients ranged from 4 to 14 years and the mean age at referral was 7.8 years. There were 7 females and 6 males. All patients were younger than 16 years old. In our study, symptoms of patients exhibited lower back pain, leg pain and urinary incontinence (hyperreflexic and hypertonic bladders were detected during urodynamic studies). We also performed (SSEP) for spinal cord conduction in all patients. There was poor cooperation for the
examination in two toddlers. Greater than 50% decrease in amplitude or a >10% increase in latency was considered as pathological. All SSEP recordings were done at lumbar, thoracal, cervical levels and the somatosensorial cortex simultaneously. Magnetic Resonance Imaging (MRI) scans were performed in all patients. Normal conus medullaris level was accepted as between the lumbar 1- and 2-disc spaces and the normal thickness of filum terminale as taken as <2 mm in Group I. Low conus medullaris level was accepted as L3 below and abnormal thickness and fatty of filum terminale in Group II (Table 1). All cases underwent a standard surgical procedure of a single level lumbar hemilaminectomy at the fifth lumbar vertebrae on the left side.

Three groups were formed for the study. Control group (n 4); FT samples obtained from cadaver, Group I (n 6); normal appearance FT samples obtained at surgery from patients with TCS, Group II (n 7); anormal appearance FT samples obtained at surgery from patients with TCS. There was no congenital malformation or disease of the central nervous system in the adult cadavers without tethered spinal cord and patients. BDNF, GFAP, Nestin, Noggin expression was demonstrated using immunohistochemistry technique in the samples obtained from all three groups. For immunohistochemical examinations, sections (5 µm) were deparaffinized in xylene and after being rehydrated by passing through decreased alcohol series, they were washed with distilled water for 10 min. Tissue sections were subsequently incubated in citrate buffer (pH: 6.0) (Lab Vision, Thermo Scientific, Fremont) and 3% hydrogen peroxide (Genemed Biotechnology, Hannover, Germany). Ultra V block (Genemed Biotechnology, Hannover, Germany) was applied for blocking. Tissue sections were incubated with BDNF (Santa cruz, CA, USA), Nestin (Santa cruz, CA, USA), GFAP (Thermo, CA, USA), Noggin (Santa cruz, CA, USA) primer antibody in 1:100 dilution for 1 h. Then, the tissue sections were incubated with secondary antibody (Genemed Biotechnology, Hannover, Germany) for 10 min. The reaction was revealed by streptavidin peroxidase complex (Genemed Biotechnology Hannover, Germany) with Diaminobenzidine (DAB). Mayer’s haematoxylin was used for background staining. The slides were evaluated by 2 independent investigators under light microscope (Olympus, Tokyo, Japan). In the staining, the H score was calculated according to the intensity of the involvment and the percentage of involvement amount by randomly selecting five areas in each preparation in x400 magnification. Retention intensity was scored as semi-quantitative 0 (0, no involvement), 1 (+, weak immunoreactivity), 2 (+ +, moderate immunoreactivity), 3 (+ + +, strong immunoreactivity). With proportioning the cell constructs which consists of retention amount percentage immunoreactivity to the total cell structure, it was scored as 1 (0% to 10% is focal), 2 (11% to 50% is regional) and 3 (51% to 100% is diffuse). The intensity and quantity scores obtained for each area were calculated with the formula of Σ Pi.(i+1) (Pi: Percentage of retention amount; i: Retention intensity). The results were collected and it was reached to a single value for that slide. The obtained data were evaluated by SPSS (Statistical Package of Social Science) Software 15.0. The histological values of the study groups, the number of percent and the averages were taken and Kruskall Wallis analysis was performed through the nonparametric tests.

**Results**

The Control group contained of loose collagen fibers, nerve fibers and blood vessels while intense collagen fibers in Group I samples and fat cells in Group II samples. BDNF showed a strong reaction in the control group, especially in the ependymal cells surrounding the central canal, while the collagen fibers and neuroglia were moderate.

BDNF immunoreactivity was moderate in Group I samples and weak in Group II samples. The results of the patient groups showed a statistically significant decrease compared to the Control group (p<0.05), and a significant difference was observed between the Group I and Group II samples (p<0.05) (Figure 1, Table 2).

While GFAP immunoreactivity was found to be strong in the Control group, especially in central ependymal cells and neuroglia surrounding the central channel, it was seen that there was varying severity in the Group I samples. When these results were compared, a statistically significant decrease in GFAP expression of Group I samples was found (p<0.05). In Group II samples, it was seen that GFAP immunoreactivity was widespread and strong in central canal and neuroglia’s and a significant increase (p<0.05) compared to Control group (Figure 2, Table 2).

Nestin immunoreactivity was occurred to be mild in the Control group and weak in Group I and Group II samples. There was a significant decrease in Nestin expression compared to the Control group (p<0.05), but the difference between the patient groups was
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Table 1: Details of patients.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gender</th>
<th>Age (Years)</th>
<th>Clinic symptoms</th>
<th>Radiological findings</th>
<th>Urodynamic findings</th>
<th>SSEP findings</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2 M/4 F</td>
<td>04-13</td>
<td>Lower back pain, leg pain and urinary retention or incontinence (one or more)</td>
<td>Normal level conus medullaris, normal thickness of filum (&lt;2 mm)</td>
<td>Hyperreflexic and hypertonic bladder</td>
<td>Normal</td>
</tr>
<tr>
<td>II</td>
<td>4 M/3 F</td>
<td>04-14</td>
<td>Lower back pain, leg pain and urinary retention or incontinence (one or more)</td>
<td>Low level conus medullaris, abnormal thickness and fatty filum terminale</td>
<td>Hyperreflexic and hypertonic bladder</td>
<td>Blocked lumbar, thoracal, cervical levels and the somatosensory cortex</td>
</tr>
</tbody>
</table>

Table 2: BDNF, GFAP, Nestin and Noggin HSCOR levels of filum terminale tissues in Control, Group I and Group II.

<table>
<thead>
<tr>
<th></th>
<th>Control groups (Cadavra)</th>
<th>Group I TCS</th>
<th>Group II TCS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Central canal</td>
<td>Collagentissue Mean ± sd</td>
<td>Neuroglia</td>
</tr>
<tr>
<td>BDNF</td>
<td>234 ± 6.3</td>
<td>150.8 ± 3.8</td>
<td>138 ± 8.2</td>
</tr>
<tr>
<td>GFAP</td>
<td>234 ± 8.3</td>
<td>36,8 ± 5.4</td>
<td>203.4 ± 13.9</td>
</tr>
<tr>
<td>Nestin</td>
<td>160 ± 4.4</td>
<td>32.6 ± 3.9</td>
<td>146 ± 3.1</td>
</tr>
<tr>
<td>N Noggin</td>
<td>207.6 ± 3.8</td>
<td>84.6 ± 3.8</td>
<td>160 ± 3.1</td>
</tr>
</tbody>
</table>

p<0.05 (Kruskall Wallis test)

Figure 3: Nestin immunostaining (a) Control group, (b) Group I, (c) Group II. (1) = x40; (2,3) = x400; ●: Ependymal cell; →: Collagen fiber; ▲: Fat cell

Figure 4: Noggin immunostaining (a) Control group, (b) Group I, (c) Group II. (1) = x40; (2,3) = x400; ●: Ependymal cell; →: Collagen fiber; ▲: Fat cell

Discussion

The Filum Terminale (FT) is a fibrous band extending from the conus medullaris to the upper of the coccyx. It anchors the spinal cord and prevents damage from movement. The filum terminale should be elastic. The main source of spinal cord elasticity may possibly be elastin and collagen in the pia and blood vessels. Degradation of viscoelasticity causes to tethered cord syndrome associated with tension in conus medullaris [4,5]. Pathological cases of the neural tube such as the meningoceles, lipomyelomeningoceles, myelomeningoceles, split cord malformation, dernal sinus tract may be the reason of strain of filum. The stretched filum terminale can be fatty and thick and in normal thickness as well [6]. The normal structure of filum terminale has always attracted the attention of scientists. The histological and ultrastructural features of filum terminale have been investigated since the second half of the 20th century [2,7-9]. González-Robles and Glusman [8] demonstrated the presence of glial cells and nerve fibers in the FT using both light and electron microscopy. Choi et al. [4,10] described ependymal cells in the central canal which is in filum terminale, in other animals and under pathological conditions described previously. George et al. [11] found that caudal neural tube growth predictor molecules H4C4 (CD44) and NOT1 differ significantly in comparison to normal samples in a study that examined filum terminale immunohistochemistry and suggested that this symptom could cause a change in cell structure within the filum terminale, which might create a predisposition to tension. In another study by Selcuki et al. [12,13] although there are normal thickness and normal appearance of the filum terminale, it has been reported that a large amount of intense collagen fibers and hyalin and large capillary veins were detected histopathologically in patients with symptoms of tethered cord syndrome. Investigators said that the filum terminale, which should have an elastic structure, lost its elasticity due to intense collagen and hyalinization, for this reason, more tension effects were introduced on the conus and there were problems in the conus medullaris study. In another study by Fontes et al. [5] in which ultrastructural analysis of filum terminale is performed, they indicate that there is a considerable amount of elastic fiber within the normal filum terminale and indicate that the elastic properties of the filum terminale may be lost by replacing these elastic fibers with different tissues. Yamada et al. [6] suggested that

not significant (p>0.05) (Figure 3, Table 2).

Noggin immunoreactivity was found to be strong in the Control group, whereas it was moderate in Group I and Group II samples. There was a significant decrease in Noggin expression compared to the Control group (p<0.05), but the difference between the patient groups was not significant (p>0.05) (Figure 4, Table 2).
the main event that caused symptoms in thick filum terminale cases was ischemia caused by tension. In this study, it has been shown that there is a change in blood flow with tension, the reduction/oxidation rates of cytochrome oxidase were change. This rate change was evaluated as a problem in oxidative metabolism and it was reported that the problem at mitochondrial level caused structural damage in the cell [6]. In the literature, besides the normal structure of FT, both microscopic and macroscopic structural changes are defined histopathologically [8].

However, molecular studies that describe normal filum in terms of neuronal cell markers and show changes in filum terminale cases are not sufficient. In our study, we evaluated 2 different patient groups of TCS in terms of BDNF, GFAP, Nestin and Noggin proteins which are neuronal cell markers and compared them with control filum terminale.

BDNF is a member of the family of neurotrophins involved in the survival, growth and function of neurons in the central and peripheral nervous systems. BDNF expression and concentration change in physiological events and pathological conditions. While BDNF primarily helps the neurons to develop and replenish themselves in the central nervous system, it also contributes to the structurally healthy nature of important nerve pathways and their maintenance. Scientific studies showing that BDNF plays a role in neurodegenerative diseases in recent years indicate that BDNF can be used as a new marker and therapy for these diseases [14,15]. BDNF is also an important survival factor for spinal motor neurons [16] and one of the most important drivers of neurite outgrowth [9]. Also, it has been well demonstrated, in rat acute and chronic Spinal Cord Injury (SCI) model, that immediate administration of BDNF in the SCI model promotes significant rubrospinal axonal regeneration and prevents axotomy-induced atrophy and/or death of rubrospinal neurons [17]. Previously studies showed that the three neurotrophins (NGF, BDNF, and/or NT-3) the potential differential effects of the on during development. A developmentally dependent susceptibility of sensory neurons to NGF and BDNF has been previously demonstrated and at certain early stages of development the application of both NGF and BDNF to cultured dorsal root ganglion cells shows an additive effect [18]. In our study, we found that BDNF expressed in control FT decreased significantly in patient groups and this decrease was higher in Group II patient. In our study, TCS suggested that neuron suppression might lead to a decrease in BDNF expression in Group I and Group II.

Glial Fibril Acidic Protein (GFAP) is an intermediate filament protein expressed by all cell types of the Central Nervous System (CNS), including astrocytes and ependymal cells during development. It is involved in many important CNS processes, including cell communication and the functioning of the blood brain barrier. Changes in GFAP level may result in disruption of the link between neuron-neuron and neuron-glia [19-21]. Rather, abundant glial cells and neurons are present in the normal filum [4]. This study confirmed the presence of GFAP immunoreactivity in glial tissue, ependyma and peri ependymal tissue within control fila and tethered fila. In our study, we observed that GFAP was expressed in ependymal cells and neurogliia’s around the central canal in normal FT and decreased in Group I and increased in Group II. The detection of this glial response at the relatively late time point after the tension may reflect a direct response to motoneuron death.

Nestin is an intermediate filament protein that regulates the cytoskeleton. Nestin is also well known as a neural progenitor cell marker. In early stages of development, Nestin is not observed in adulthood which expressed by many cell types, including central and peripheral nervous system cells [22,23]. However, it has been shown that reinduction of Nestin expression in pathological conditions, such as after central nervous system damage in adults. Recently, several laboratories including our own have isolated neural progenitor cells from the FT of both rats and humans. These cells have been shown to express neural progenitor cell markers such as Nestin, Dlx-2, Sox-2, and Musashi-1. They have also been passaged multiple times as neurospheres and differentiated into neurons, astrocytes, and oligodendrocytes [24,25]. Persistent pluripotential embryonic cells that fail to regress and disappear are believed to cause abnormalities of the filum [26]. In our study, Nestin significantly decreased in patient groups, where presence in the control FT. We also believe that the decrease of Nestin in patient groups is related to the decrease of progenitor cells in the FT.

Noggin is a protein that enters into in the development of many tissues. Noggin is a signal molecule that plays an important role in promoting somite pattern in embryo development. Noggin function is required for proper nervous system, somit and skeletal development [27]. In our study, we determined that Noggin was expressed and decreased significantly in the patient groups and this decrease was higher in the Group II patient.

The presence of neuronal precursor cells in FT has been demonstrated by various methods in recent studies. The expression of these cells in neural progenitor cell markers such as Nestin, GFAP, Sox2 and Musashi-1, and their differentiation of these cells into neurons, astrocytes and oligodendrocytes as neurosphere, produced speculation that FT may contain neural stem cells [28,29].

Conclusion

In our study, we demonstrated Nestin and Noggin expressions known as neuronal stem cell markers in the control and patient groups. Accordingly, we concluded that FT, known as a fibrovascular structure with no neurogenic potential and clinical significance, contained progenitor cells (stem cell-like) in the control groups, and decreased progenitor cells by decreasing Nestin and Noggin expression in FT in TCS groups.

BDNF and GFAP expressions which were responsible for neuronal cell functions were shown in the control and patient groups. This suggests that decreased BDNF and GFAP proteins may play a role in TCS formation compared to the control group. The mechanisms by which FT has undergone thick and fat formation in TCS are not yet known. We believe that the different distribution of the molecules we have studied during the TCS formation process may be one of the explanatory reasons for the underlying mechanisms of TCS formation.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards. Ethics committee approval was obtained from the Manisa Celal Bayar University School of Medicine No: 20478486-24.

References


