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Myelin basic protein profile of central nervous system in experimentally induced demyelination and remyelination

[Deneysel demiyelinasyon ve remiyelinasyonda merkezi sinir sisteminin miyelin temel protein profili]

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ABSTRACT

Objective: The aim of the present study was to assess myelin basic protein (MBP) profiles of central nervous system in experimentally induced demyelination and remyelination.

Materials and Methods: Sixty 8-10 weeks old male C57Bl/6 mice were used for this study. Twenty mice were selected as control and 40 mice as experimental group. Experimental group was fed *ad libitum* with a 0.3 % cuprizone diet for 6 weeks. At the end of 6 weeks, 20 mice from the experimental group and 10 mice from the control group were euthanasied and the remaining of the experimental group was fed cuprizone-free diet for more 4 weeks and then 10 mice in the control group and 20 mice in the experimental group were sacrificed. Corpus callosum sections were removed and protein extraction was performed in all samples. The MBP profiles were evaluated by sodium dodecyl sulphate - polyacrylamide gel electrophoresis and Western-blot analysis.

Results and Conclusion: Decreased intensity of the MBP isoforms below the 26.6 kDa was observed in demyelinated group as compared with controls. In the remylinated group, intensity of MBP approached to values of control group. This study will contribute to the understanding of the mechanisms of demyelination and remyelination as well as will serve a base for the future studies investigating diseases characterized by demyelination in human and animals.

Key Words: demyelination, mice, myelin basic protein, remyelination

Conflict of Interest: Authors declare no conflict of interest.

ÖZET

Amaç: Bu çalışmanın amacı, deneysel demiyelinasyon ve remiyelinasyonda merkezi sinir sisteminin miyelin temel protein (MBP) profilini değerlendirmektir.

Gereç ve Yöntemler: Bu çalışmada 60 adet, 8-10 haftalık erkek C57BL/6 fare kullanıldı. Farelerden yirmi adeti kontrol grubu, 40 adeti deneme grubu olarak ayrıldı. Deneme grubundaki fareler 6 hafta boyunca % 0.3 kuprizon içeren diyet ile beslendi. Bu süre sonunda, deneme grubundan 20 fare ile kontrol grubundan 10 farenin ötanazileri yapıldı. Deneme grubundaki diğer 20 fare ile kontrol grubundaki 10 fare, remiyelinasyon oluşturmak amacıya, 4 hafta boyunca kuprizon içermeyen diyet ile beslendi ve sakrifiye edildi. Farelerin korpus kallosumları çıkartıldı ve tüm örneklerde protein ekstraksiyonu yapıldı. MBP profilleri SDS-PAGE ve Western-blot analizi ile değerlendirildi.

Bulgular ve Tartışma: Demiyelinasyon grubunda 26.6 kDa altındaki MBP izoformlarının yoğunluğunun kontrol grubuna göre azaldığı gözlendi. Remiyelinasyon grubunda MBP yoğunluğunun kontrol grubundaki düzeye yaklaştığı belirlendi. Bu çalışma, demiyelinasyon ve remiyelinasyon mekanizmalarının anlaşılmasına katkıda bulunmasının yanı sıra, insan ve hayvanlarda demiyelinasyon ile karakterize hastalıklarla ilgili çalışmalar için temel teşkil edecektir.

Anahtar Kelimeler: demiyelinasyon, fare, miyelin temel protein, remiyelinasyon.

Çıkar Çatışması: Yazarlar arasında çıkar çatışması bulunmamaktadır.

Introduction

Myelin basic protein (MBP) is a component of protein structure of the myelin sheath which is synthesized by oligodendrocytes in central nervous system [1]. MBP serves in the process of myelination for nerves in the central nervous system and the stabilization of multi-storey structure of the myelin [2]. Severe hypomyelination has been demonstrated in central nervous system neurons of shiverer mouse which was knockout for the MBP [3]. MBP has also been proposed as a sensitive marker of myelination [4]. Five different isoforms of myelin basic protein with molecular masses of 14.0 kDa, 17.22 kDa, 17.24 kDa 18.5 kDa and 21.5 kDa have been identified in murine brain [5, 6]. Fourteen kDa and 18.5 kDa isoforms have been reported to be predominantly expressed in active myelination in murine brain. The 17 kDa and 21.5 kDa isoforms have been suggested to play roles in early stage of myelinogenesis and also to be associated with remyelination [1, 8]. Studies indicate that central nervous system disorders can give rise to cerebrospinal fluid MBP concentrations [9,10]. The association between the presence of anti-MBP antibody and clinical progression of multiple sclerosis has been postulated [11].

The destruction of myelin sheath is defined as demyelination. Inflammatory events, infectious and autoimmune diseases, metabolic disorders and toxic agents lead to the myelin destruction [12]. Multiple sclerosis, Alzheimer disease, acute disseminated encephalomyelitis, progressive multifocal leukoencephalopathy and distemper are characterized by demyelination [13]. The myelin sheath is required for the conduction of nerve impulses and its destruction can lead to disruption in communication of nerve signals and dysfunctions of the nervous system. Brain functions become impaired when demyelination occurs. Studies have shown that new myelin sheaths can be restored to the demyelinated axons by endogenous repair mechanisms or by transplantation of myelinating cells, called myelin repair or remyelination [14, 15]. The formation of new myelin sheaths has been reported following demyelination in mouse model [16].

The mechanisms of demyelination and remyelination have been extensively researched both *in vitro* and *in vivo*. Repair of the myelin sheath constitutes an important part of treating demyelinating diseases. There were no sufficient reference data on the effective treatment for demyelination in the literature we reviewed. Such reference document may help better understand MBP expression in demyelination and remyelination conditions and provide guidance on the treatment in demyelinating diseases. Thus, the aim of the present study was to investigate myelin basic protein profile of corpus callosum in cuprizone-induced demyelination and remyelination.

Material and methods

Experimental procedures of demyelination and remyelination

A total of sixty 8-10 weeks old male C57Bl/6 mice were used for this study. Twenty mice were selected as control and 40 mice as experimental group. Experimental demyelination in mice was performed according to the procedure proposed by Lindner et al. [17]. Mice in the experimental group were fed *ad libitum* with a 0.3 % cuprizone (bis-cyclohexanone oxaldihydrazone) (Sigma-Aldrich Inc., St. Louis, MO, USA) diet for 6 weeks. The mice were monitored for clinical symptoms for 6 weeks. At the end of 6 weeks, 20 mice from the experimental group and 10 mice from the control group were euthanasied with a high dose of ether anesthesia. In order to check if a remyelination occurs in the absence of cuprizone the remaining of the experimental group was fed cuprizone-free diet for more 4 weeks. At the end of the experimental procedure, 10 mice in the control group and 20 mice in the experimental group were sacrificed by high dose ether anesthesia.

Histopathological analysis

The right corpus callosum of all sacrificed mice were removed and immediately fixed in 4 % formol solution and then embedded in paraffin. To evaluate the myelination in serial sections of the corpus callosum, Luxol fast blue staining method was used [18].

Tissue preparation

The left corpus callosum of all sacrificed mice were rapidly removed, weighed, and frozen at -70°C until analyses. Corpus callosum tissues were homogenized in Nonidet-P40 lysis buffer (150 mM sodium chloride, 1.0 % NP-40, 50 mM Tris, pH 8.0) using homogenizer (Bio-Gen PRO200, PRO Scientific Inc., Rd Oxford, CT, USA). Tissue homogenates were transferred to microcentrifuge tubes and then centrifuged at 10.000 x g for 10 minutes at 4° C. The supernatants were removed and the centrifugation process was repeated. The MBP profiles were evaluated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and Western-blot analysis.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein concentration of corpus callosum extracts was determined spectrophotometrically (Nanodrop-1000, Thermo) and the protein concentrations were adjusted to 15 mg/ml. The extracts were denatured by boiling at 95°C for 5 min in sample buffer [0.1 M Tris-HCl (pH 6.8) containing 20 % (w/v) glycerol, 4 % (w/v) SDS, 2 % (v/v) 2-mercaptoethanol and 0.02 % (w/v) bromophenol blue]. SDS-PAGE was used to separate proteins [19]. SDS-PAGE was carried out using vertical slab gel electrophoresis apparatus (Thermo EC 120, New York,

USA). Duplicate 12.5 % SDS-PAGE gels were prepared. Twenty ng samples were loaded onto gels. Wide range marker (Sigma-Aldrich Chemie GmbH, Germany) was loaded onto first gel and protein bands were visualized by staining with Blue silver [20]. Prestaining marker (SDS7B2, Sigma-Aldrich Chemie GmbH, Germany) was loaded onto second gel and this gel remained without staining. Silver-stained gel was destained in methanol:water:acetic acid (45:45:10). Molecular weights of proteins on gel were determined by comparing with marker protein standards and are expressed in kilodalton (kDa).

Western blotting

The fractionated proteins were transferred onto polyvinyl difluoride (PVDF) membrane at 90 mA for 45 min. and then were incubated in blocking buffer for 1 h at 4°C. The membranes were then incubated with MBP primary antibody (MBP, C-16: sc-13914, Santa Cruz Biotechnology, Inc. Heidelberg, Germany) diluted 1:100 with phosphate-buffered saline. Followed by washing, biotinylated secondary antibody and AB enzyme were applied according to the manufacturer's instruction (goat ABC staining system: sc-2023). The blots were incubated with peroxidase substrate and then molecular weights of proteins on membrane were evaluated using protein standards (SDS7B2). Visible bands were photographed.

Ethical approval

This study was approved by the Experimental Animal Studies Ethics Committee of Ondokuz Mayis University.

Results

Histopathological findings

Severe demyelination was seen in corpus callosum sections from demyelinated group. The myelination pattern of corpus callosum in remyelinated group were similar to those of the control group (Figure 1).

Electrophoretic findings

Typical electrophoretic profiles of serum proteins from experimentally demyelinated and remyelinated mice and control group are shown in Figure 2. Several protein fractions ranging in molecular weight from 14-175 kDa were observed from brain extracts in each group. Although the protein concentrations of tissues were equalized, a noteworthy decrease in all band densities were observed of the demyelinated and remyelinated mice compared with the controls.

Western blot results

Figure 3 shows the representative Western blot results of MBP from control, demyelinated and remyelinated mice. Decreased intensity of the MBP isoforms below the 26.6 kDa was observed in the demyelinated group as compared with controls. In the remylinated group, intensity of these isoforms of MBP approached to values of control group. Western blot analysis showed that there was no additional band in remyelinated group.

Discussion

Demyelinating diseases are a group of illness that are characterized by myelin loss and their aetiologies are not known completely yet. In recent years, scientific research has focused on the myelin repair in demyelinating diseases [21-24]. Experimental and clinical studies have shown that the central nervous system has capacity for myelin repair [15, 21, 25]. Cuprizone has been used extensively for studies in experimental demyelination and remyelination [15, 26, 27]. Oligodendrocytes and their myelin sheaths are susceptible to the cuprizone toxicity and its exposure leads to necrosis and apoptosis of these cells and myelin loss [15, 28]. During remyelination, oligodendroglial progenitor cells proliferate and spread within the demyelination areas and differentiate into mature oligodendrocytes [15]. Importantly, cuprizone-induced demyelination is a good model for studying the pathology and repair mechanism of multiple sclerosis [29, 30]. The different doses and durations of cuprizone

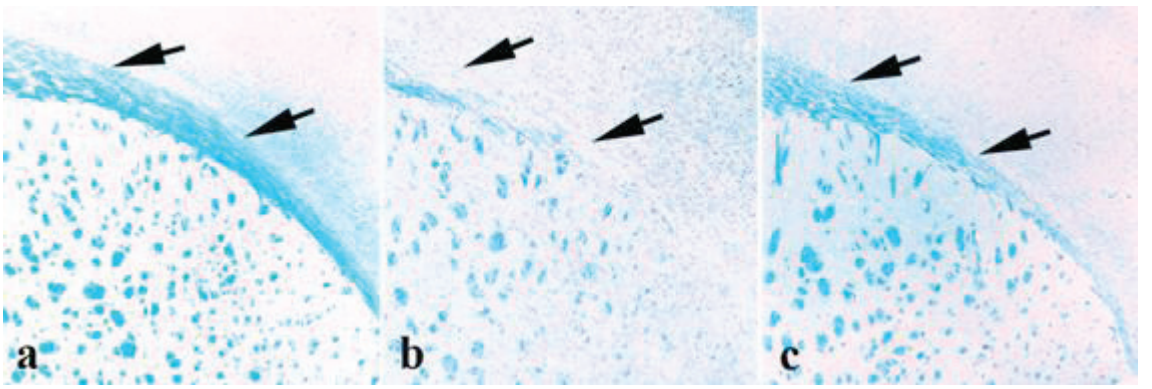

Figure 1. The patterns of myelination in the corpus callosum sections of the groups (Luxol fast blue myelin staining). a: normal myelination in the control animals, b: severe myelin loss in experimentally demyelinated mice, c: remyelination in experimentally remyelinated mice.

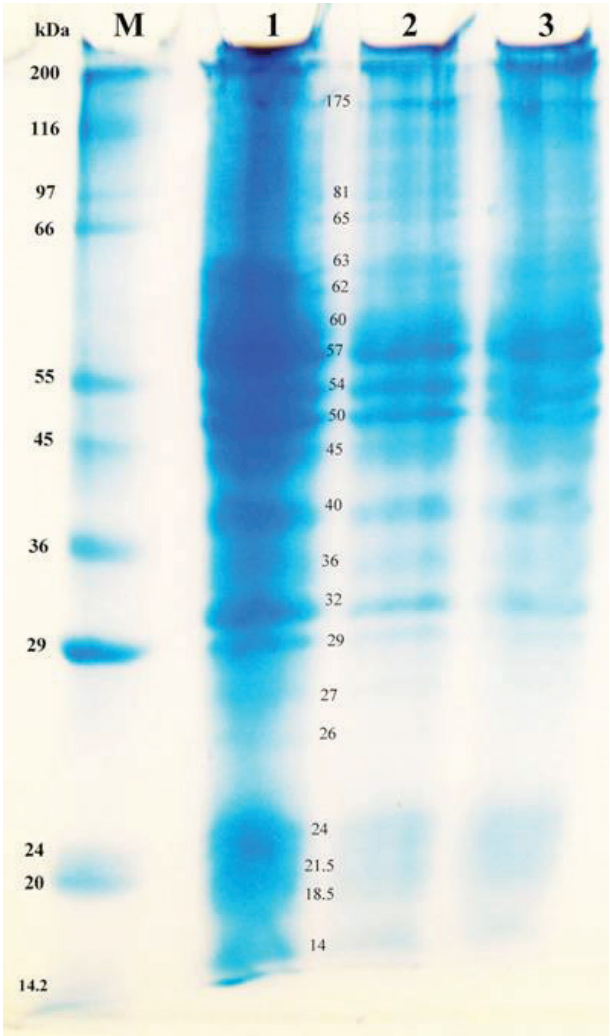

Figure 2. Typical serum protein fractions of remyelinated (1), demyelinated (2) and control (3) groups. M: Molecular weight marker.

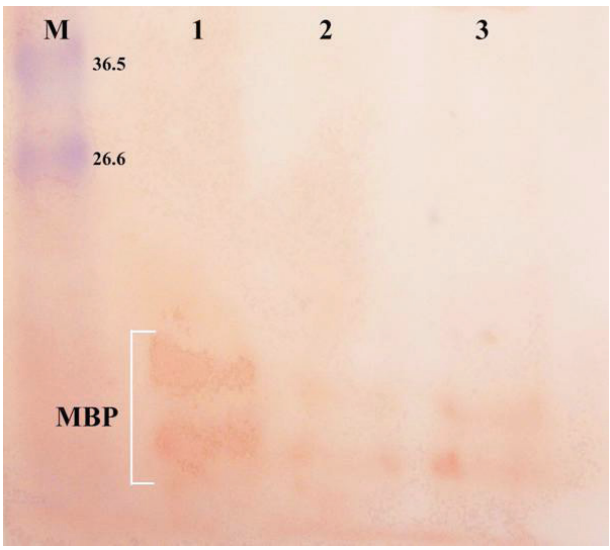

Figure 3. Western blots of myelin basic protein (MBP) in control (1), demyelinated (2) and remyelinated (3) groups. M: Molecular weight marker.

administration have been used to create demyelination and remyelination [26, 27, 31]. Inhibition of remyelination capacity in superior cerebellar peduncles of mice due to long-term administration of cuprizone has been suggested [32]. Start of remyelination has been reported one week after withdrawal of cuprizone [33]. However, new myelin sheaths on axons in remyelinated areas have been determined to be thinner than normal sheats [16, 14, 34]. In our study, severe demyelination in the corpus callosum was determined after 6 weeks of 0.3 % cuprizone administration. Our findings were consistent with Lindner et al. [17] documenting complete demyelination in corpus callosum in mice 6 weeks after 0.3 % cuprizone exposure.

It has been well documented that the conduction of nerve impulses and normal functioning of the nervous system are mainly dependent on myelination [35, 36]. Understanding of the mechanism of myelin sheath formation and destruction is crucial for therapeutic management of demyelinating diseases. MBP has been suggested to be a sensitive marker of myelination [4]. Detectable levels of MBP in cerebrospinal fluid closely correlated with clinical activity of multiple sclerosis have been reported [37]. Altered MBP levels have been observed in CSF following different types of neurodegenerative disorders. Increased levels of MBP in cerebrospinal fluid have also been reported in patients with multiple sclerosis [10]. Relationship between the immunoreactivity of MBP in CSF and the destruction of nervous tissue has been demonstrated in patients with encephalitis [9]. Recently, Massella and co-workers [38] demonstrated down-regulation of MBP mRNA expression in experimental allergic encephalomyelitis in rats which is the disease model for multiple sclerosis.

Numerous studies have focused on expression of MBP in cuprizone-induced demyelination and remyelination immunohistochemically. Ludwin and Sternberger [39] demonstrated immunohistochemically a loss of MBP in the superior cerebellar peduncles of mice with cuprizone-induced demyelination and in remyelination stage; MBP expression level has been shown to be similar to that seen during normal development. Additionally, Lindner et al. [17] demonstrated that MBP expression is down-regulated in corpus callosum of mice with cuprizon-induced demyelination. They also reported re-expresion of MBP two weeks after withdrawal of cuprizone. Millet et al. [40] reported that cuprizone administration to CNP::EGFP transgenic mice resulted in decreased immunoreactivity of MBP in corpus callosum. However, no reference data on MBP profile in the corpus callosum of mice with cuprizone-induced demyelination and remyelination were available in the literature we reviewed. In the present study, SDS-PAGE profiles revealed that the percentages of several protein fractions ranging from 14 kDa to 175 kDa in demyelinated and remyelinated groups were lower

density than that of control group. Western blotting showed decreased intensity of MBP isoforms below 26.6 kDa in demyelinated group as compared with controls. Decreased intensity of MBP isoforms in mice with demyelination may be associated with the oligondendrocyte death resulting cuprizone toxication. In the remylinated group, intensity of MBP isoforms approached to values of control group. Previously, the 17 kDa and 21.5 kDa isoforms were shown to be associated with remyelination [7]. Our results demonstrate alterations in the myelination associated with alterations in MBP profile in corpus callosum.

The lower intensity of MBP in the corpus callosum in cuprizone demyelination reconfirms results from immunohistochemical studies [17, 39, 40]. This study will contribute to the understanding of the mechanisms of demyelination and remyelination, as well as will serve a base for the future studies investigating diseases characterized by demyelination in human and animals.

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Conflict of Interest: Authors declare no conflict of interest.

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