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ORIGINAL ARTICLE

Changes in levels of oxidative stress markers and some neuronal enzyme activities in cerebrospinal fluid of multiple sclerosis patients

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Abstract

OBJECTIVES: The aim of the present study was to assess cerebrospinal fluid (CSF) levels of malondialdehyde (MDA), F2 isoprostanes (8-iso-$PGF_{2\alpha}$) and total antioxidant status (TAS) in relapsing-remitting (RR) and secondary progressive (SP) course of MS and neurological controls. These parameters were correlated with brain tissue damage parameters – neuron-specific enolase and 3´,5´-cAMP-phosphodiesterase (PDE) in CSF.

METHODS: CSF samples were obtained from MS patients divided into two groups according to the disease severity (EDSS) – RR and SP course of MS. Control group composed of neurological diagnoses without demyelination and neurodegeneration. 8-iso-$PGF_{2\alpha}$ and NSE levels in the CSF samples were determined using specific immunochemistry assays. MDA levels in the CSF were measured by HPLC method after reaction with thiobarbituric acid in acidic conditions. TAS and total PDE activity of CSF was determined spectrophotometrically.

RESULTS: There were significant differences in CSF MDA levels between MS group and controls and also between RR and SP disease course. By contrast, CSF levels of 8-iso-$PGF_{2\alpha}$ in MS group and both forms of MS were comparable to control values. In addition, the results show increased CSF levels of PDE in MS group and no changes of NSE in CSF between MS and control group.

CONCLUSION: These findings point to a possibility of using the parameters of different specificity to lipid peroxidation for monitoring different stages (acute/progressive) of MS. This study support the idea, that combination of CSF markers is important for monitoring overall brain tissue pathology in MS.

Abbreviations:

cAMP	- cyclic adenosine monophosphate
CNS	- central nervous system
CSF	- cerebrospinal fluid
MDA	- malondialdehyde
MS	- multiple sclerosis
NSE	- neuron-specific enolase
PDE	- 3',5'-cAMP-phosphodiesterase
RR MS	- relapsing-remitting MS
SP MS	- secondary progressive MS
TAS	- total antioxidant status
TBARS	- thiobarbituric acid- reactive substances
8-iso-$PGF_{2\alpha}$	- 8-isoprostane-prostaglandine $F_{2\alpha}$

INTRODUCTION

Multiple sclerosis (MS), the most common cause of nontraumatic disability in young adults, is a chronic complex neurological disease with a variable clinical course and several pathophysiological mechanisms such as inflammation, demyelination, axonal/neuronal damage, gliosis, oligodendrocyte loss, remyelination and repair mechanisms, and oxidative stress (Glass *et al.* 2010; Durfinova *et al.* 2014). Central nervous system (CNS) is sensitive to oxidative damage, due to the high rate of oxygen utilization and relatively poor antioxidant defense system (Mattsson *et al.* 2007), which is considered to be an important in both acute and chronic neurodegenerative diseases (Floyd & Carney 1992). In patients with multiple sclerosis (MS), oxidative stress is associated with significant damage to myelin and axons, which in turn leads to clinical symptoms associated with progressive loss of axons and other brain structures damage (van Horssen *et al.* 2011). Cells protect themselves against oxidative stress via an antioxidant defense system, which utilizes free radical scavengers and other enzymes to maintain the appropriate redox state of cellular proteins. These free radical scavengers and antioxidant enzymes include superoxide dismutase, catalase, glutathione peroxidase, and the thiol tripeptide glutathione. In the inflammatory state or during the failures of antioxidant mechanisms, after overproduction of reactive oxygen/nitrogen species, these can cause damage of lipids, proteins, and nucleic acids and may lead to cell death. Appropriate candidates for free radical attack in brain are myelin sheet membranes rich in polyunsaturated fatty acids.

MDA is a three-carbon dialdehyde resulting from lipid peroxidation, primarily arachidonic acid metabolism (Uchida 2000), which is formed during myelin oxidation *in vitro* (van der Veen & Roberts 1999) and in the CNS during experimental autoimmune encephalomyelitis (Espejo *et al.* 2002). Previously, MDA, produced from the decomposition of the unstable peroxides derived from polyunsaturated fatty acids, has been extensively used as a lipid peroxidation marker by measuring levels of thiobarbituric acid-reactive substances (TBARS; Lovell *et al.* 1995; Mir *et al.* 2014; Wang *et al.* 2014; Kaneda *et al.* 2010). However, the validity of this method is limited because it also measures other aldehydes conjugated to TBARS, as well as nonlipid related chromogens.

Isoprostanes are a class of lipid peroxidation products that are generated when free radicals attack the arachidonic acid esterified in phospholipid pools of cell membranes. They offer advantages over indices of lipid peroxidation. For instance, in contrast to lipid hydroperoxides, which rapidly decompose, isoprostanes are chemically stable end-products of lipid peroxidation that are released by phospholipases, circulate in plasma, and excreted in urine (Morrow *et al.* 1992). 8-Iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is one of the most abundant and well-recognized isoprostanes and is now recognized as a "gold-standard biomarker for *in vivo* oxidative stress and lipid peroxidation" (Fam & Morrow 2003; Mir *et al.* 2014).

Myelin degenerates in Wallerian degeneration that follows traumatic injury to axons. If not removed, which is the case in the injured CNS, degenerated myelin inhibits axonal regeneration (Filbin 2003). Myelin removal is executed by microglia and macrophages via receptor-mediated phagocytosis through cAMP cascade (Smith 2001; Makranz *et al.* 2006). Balance between G_s-controlled cAMP production by adenylyl cyclase and cAMP degradation by PDE sustains normal operating cAMP levels that enable efficient phagocytosis (Makranz *et al.* 2006).

NSE is a glycolytic enzyme that is localized primarily in the neuronal cytoplasm. In adults, CSF concentrations of NSE have served as markers of neuronal damage in patients with a variety of neurological conditions (Pollak *et al.* 2003).

The present study aims to evaluate isoprostane (8-iso-$PGF_{2\alpha}$) concentration in the CSF of patients with MS and to investigate its association with level of malondialdehyde (MDA), total antioxidant status (TAS) and neuronal damage parameters: 3',5'-cAMP-phosphodiesterase (PDE), neuron–specific enolase (NSE).

MATERIAL & METHODS

Patients

The study involved 90 participants divided into the two groups. All MS patients (57 subjects) were diagnosed at the 2nd Department of Neurology, Faculty of Medicine in Bratislava, and divided to two groups according to McDonald criteria (Polman *et al.* 2011): relapsing-remitting (RR) and secondary progressive (SP) course of the disease. Control group included 33 patients with non-demyelinated neurologic disorders (R51, H81, H82, M54, M33, M50, F41, S72 and K74).

Methods of evaluation

CSF samples were obtained by standard lumbal puncture. After centrifugation at 4 000 rpm for 10 min. aliquots of CSF were stored at –80 °C until use. 8-iso-$PGF_{2\alpha}$ and NSE levels in the CSF samples were determined using a specific competitive EIA kits from Cayman Chemical. MDA levels in the CSF were measured after reaction with thiobarbituric acid in acidic conditions according to Ohkawa *et al.* (1979) by HPLC. TAS in the CSF was determined by the TEAC (Trolox-equivalent antioxidant capacity) method according to the Re *et al.* (1999). Total PDE activity was measured spectrophotometrically according to Cheung (1967). This study was approved by the Ethics Committee to a project and all patients gave their informed consent to participate.

Statistics

StatsDirect version 2.7.2 was used for statistical analysis. Statistical significance was set at p-values ≤0.05. Differ-

ences between disease groups in CSF were analyzed by non-parametric Mann-Whitney test.

RESULTS & DISCUSSION

The current MS patients and control group were matched by age, sex and MS patients also by EDSS scale (Kurtzke 1983; Table 1). Age of subjects in control group was significantly higher than in total MS and RR-MS group, respectively (p=0.0004; p<0.0001). This intergroup variability could be associated with the low age of subjects in time of diagnostic. EDSS was significantly higher in SP-MS group in comparison to total MS and RR-MS patients (p=0.0008; p<0.0001).

There were significantly increased CSF levels of MDA in MS patients in comparison to control group of neurological patients (p=0.05; Table 2). MDA production in CSF was monitored *in vitro* by adding different activators of lipoperoxidation to CSF within 30 minutes. As activators, the mixture of Fe (125 pmol/ml) + ascorbate (3.125 nmol/ml), Fe (125 pmol/ml) and temperature of 40 °C were used. There were not observed any significant elevations in CSF MDA levels after using these activators, which may pointed to an absence of substrates for the production of MDA in isolated CSF (unpublished results). The range of MDA levels varied with MS forms: MDA levels was significantly higher in patients with SP-MS form than in patients with RR-MS form (p=0.0059; Table 3). Similarly patients with higher EDSS had higher MDA levels in CSF and plasma in study of Ljubisavljevic *et al.* (2013). These results were not in correspondence with specific parameter of lipid peroxidation in the brain – isoprostanes (Mir *et al.* 2014). Contrary to the literature data (Greco *et al.* 1999; Mattsson *et al.* 2007), the mean value of 8-iso-PGF_2 levels of both forms of MS patients were comparable with 8-iso-PGF2 levels in a control group in CSF (Table 2).

Our results showed no correlation between MDA and 8-iso-$PGF_{2\alpha}$, as the oxidative stress parameters. These results might point to different ways of their production. Unlike isoprostanes, MDA is considered a non-specific parameter of lipid peroxidation and the TBARS assay is not specific for MDA (Halliwell 2000). F_2-isoprostanes can be produced also by inducible form of cyclooxygenase-2 (COX-2) from arachidonic acid during inflammation (Minghetti 2004). The most abundant polyunsaturated fatty acid in CNS is docosahexaenoic acid (C22:6ω3). Oxidation of this 22-carbon fatty acid may result in the formation of isoprostane-like compounds, termed neuroprostanes (e.g. F_4 neuroprostanes), which may be unique markers of oxidative neuronal injury (Roberts *et al.* 1998). Published studies revealed significant differences in the oxidative stress intensity indices in patients with different clinical forms and phases of MS (Lutsky *et al.* 2014). No changes were determined in 8-iso-PGF_2 levels with increasing progression of disease in our patients, but there were significantly higher MDA levels in SP-MS vs. RR-MS according to literature (Ljubisavljevic *et al.* 2013). This finding could indicate that 8-iso-PGF_2 might be also the parameter of inflammatory processes in the early state of disease and MDA is more likely the parameter for monitoring the disease progression. MS group could be also divided according to treatment conditions because COX-2 expression has been showed to be down-regulated by glucocorticoids (Yamagata *et al.* 1993).

Production of F_2 isoprostanes is modulated by TAS (Roberts & Morrow 2000) and in our conditions, TAS

Tab. 1. Demographical characteristics of patients and control group.

	Control	total MS	RR-MS	SP-MS
age (years)	57	41***	40***	37
	(48-62)	(31-53)	(29-50)	(35.5-53)
male/female	21/12	25/32	21/29	4/3
EDSS	–	2.5	2	4××
		(2-3.5)	(2-3)	(4-4)
n	33	57	50	7

Values are expressed as median with an interquartile range (Q1-Q3; 25–75%). *** Significantly different from control group p≤0.0005; ×× significantly different from total MS and RR-MS group.
EDSS – Expanded Disability Status Scale, MS – multiple sclerosis, RR-MS – relapsing-remitting MS, SP-MS – secondary progressive MS

Tab. 2. Measured values of CSF parameters of MS patients and control group.

Parameter	MDA (nmol/ml)	Iso-PGF_2 (nmol/ml)	TAS (μmol/ml)	PDE activity (pcat/ml)	NSE (ng/ml)
Controls	0.6	19.69	0.22	19.8	4.67
	(0.43–1.07)	(12.33–30.21)	(0.16–0.25)	(7.67–37.7)	(3.4–6.82)
n	18	12	14	17	12
MS	1.23 *	12.67	0.27	38.8	5.11
	(0.58–2.39)	(8.75–22.2)	(0.19–0.32)	(15.02–64.45)	(3.87–6.57)
n	34	23	29	31	27

Values are expressed as median with an interquartile range (Q1-Q3; 25-75%). * Significantly different from control group p≤0.05.

Tab. 3. Measured values of CSF parameters of relapse-remitting and secondary progressive MS groups.

Parameter	MDA (nmol/ml)	Iso-PGF_2 (nmol/ml)	TAS (μmol/ml)	NSE (ng/ml)
RR-MS	1.13	12.58	0.27	5.11
	(0.56–1.74)	(5.87–22.15)	(0.2–0.31)	(3.82–6.64)
n	29	21	26	21
SP-MS	3.27 **	12.67	0.18	5.12
	(2.5–3.85)	(11.17–19.34)	(0.16–0.38)	(4.37–5.92)
n	4	3	3	6

Values are expressed as median with an interquartile range (Q1–Q3; 25–75%). ** Significantly different from RR-MS group $p \leq 0.005$.

was comparable in CSF of the both MS and control groups (Table 2), also of RR-MS and SP-MS patients (Table 3). These values are comparable with study of Ghabaee *et al.* (2010). Supplementation with vitamins preferred by patients is an appropriate supportive therapy and could affect the CSF levels of isoprostanes in our patients in case of blood-brain barrier damage.

Additionally, we found a trend towards significance in enzyme activity from metabolism of cAMP (PDE) in CSF in our group of MS patients compared to the control group (p=0.0551; Table 2). We didn´t observe any differences in the levels of specific neuronal protein (NSE) from the control group (Table 3). NSE values obtained are similar to those published by Mitosek-Szewczyk *et al.* (2011). Similarly no differences between controls and patients with the first event indicative for MS were observed in study of Hein *et al.* (2008). Differences in results of the authors could be coherent with an actual status of patient during the withdrawal. RR patients whose CSF samples were used in our study were in clinically stable phase (remission). In the pathogenesis of MS occurs damage and destruction not only neurons but other brain structures (glial cells), too. This fact is confirmed by significant changes of PDE in MS group in comparison to control group in CSF. Astrocytes, which play an important role in pathogenesis of many neurodegenerative disorders (De Keyser *et al.* 2008), could participate also on progressive axonal losses in MS (Durfinova *et al.* 2014). They expressed wide variety of receptors for various neurotransmitters, including β_2-adrenergic receptors that are coupled with synthesis of cAMP through adenylate cyclase. The area of origin of the enzyme produced during the CAMP degradation (PDE) could be situated not only in neurons but also in astrocytes.

Our study was significant as it investigated the values of oxidative stress parameters as prognostic in monitoring of neurological impairment development in MS. The results presented in this study exemplify the interpretation that the pattern of different markers estimated in CSF can together reflect ongoing disease processes in the light of pathological status not only of neurons but other e.g. glial cells too.

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