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Free radical peroxidation products in cerebrospinal fluid and serum of patients with multiple sclerosis after glucocorticoid therapy

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Abstract

Multiple sclerosis (MS) patients were found to have elevated thiobarbituric acid reactive material levels, increased soluble sulfhydryl groups and reduced protein sulfhydryl groups in cerebrospinal fluid and serum, and slightly reduced superoxide dismutase in serum, which suggested disease activating free radical peroxidation. Moreover, levels of these varied across methylprednisolone (MP) therapy. We observed significant differences in the levels of peroxidation products between MS patients and controls. These changes were most evident in relapse. After MP therapy, levels of these indicators approached control values, especially in the remission period. Our findings suggest that MP protects against free radical attack.

Key words: multiple sclerosis, glucocorticoid therapy, methylprednisolone, free radical peroxidation.

Introduction

Multiple sclerosis (MS) is a disabling, inflammatory and demyelinating disease of the central nervous system (CNS) of unknown aetiology. MS is characterised by recurrent events of autoimmune-mediated demyelination and axonal loss. The disease presents different immunological pictures, clinical courses and radiological images [6,34]. Oxidative stress, which is usually defined as a preponderance of the production of free radicals over their elimination, is commonly implicated in the development of brain damage and reactive oxygen species (ROS) contribute to several mechanisms underlying the pathogenesis of MS lesions [7]. Because free radical peroxidation alters the structure of biological membranes, and thereby affects their physical and chemical properties such as permeability, resorption or potential, it can be expected to play an important role in the pathomechanism of MS [14]. Under normal conditions, free radicals are involved in a chain of various reactions necessary for regular cell functioning. Among biochemical mechanisms responsible for the toxic effects of free radicals the major one is lipoxygenation. Lipid endoperoxides resulting from lipoxygenation are not durable, and

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during their degradation subsequent free radicals and malondialdehyde (MDA) are produced [12,33]. Lipoxygenation and protein alterations are involved in biomembrane configuration abnormalities and the ensuing modification of its flow and permeability. These activated oxygen species are believed to be detoxified by cellular antioxidative enzymes such as superoxide dismutase (SOD) [10,11,31]. Non-protein thiols are important factors of the cellular defense against free radicals [44], whereas protein thiols are determined by the structure and function of many proteins [29]. Non-protein brain thiols consist mostly of reduced glutathione (GSH) under normal circumstances [35,39]. In MS, free radical peroxidation can be included as a possible pathogenic factor.

In the past few years, the role of glucocorticoids (GC) in MS relapse treatment has strengthened [23]. Their strong anti-inflammatory action makes their use the most effective method for overcoming and shortening the duration of neurological symptoms during relapses. GC activity consists mainly of the induction of specific, inflammation suppressing genes and repression of genes coding proteins which are engaged in immune and inflammatory reactions [1,22,32]. It comes to rapid inhibition of inflammatory reactions, sealing the blood-brain barrier and decreasing the magnetic resonance imaging (MRI) number of gadolinium up-taking foci [41]. Moreover, an attempt was made to determine the relationship between these biochemical characteristics of MS patients and remission of disease after methylprednisolone (MP) treatment.

The aims of this study were to verify the hypothesis that free radical peroxidation might be one of the factors implicated in the pathophysiology of MS and find out whether these biochemical parameters change after GC therapy.

Material and methods

Sample studied

Participants in the study were patients admitted to the Neurology Department of the Medical University of Lublin. Thirty seven patients (20 female and 17 male) with a mean age of 35.2 ± 5.3 with RRMS (according to the criteria in McDonalds' *et al*. [24]) were consecutively studied during relapse. Relapse was defined as a worsening on the Expanded Disability Status Scale (EDSS) by 1.0 point, new clinical symptoms of subjective character or objectively existing, lasting at least 24 hours, in the absence of infection or fever, after a period not shorter than 30 days of neurological status stability. The patients were evaluated at three different time-points, at relapse, after five days of IVMP following a dose of 1.0 g/day and in the remitting period. All 37 patients had white matter lesions on brain MRI scans. Clinical disease severity was scored by Kurtzke's EDSS [18]. The mean time for duration of clinical symptoms was 6.4 years (± 5.1). The mean number of relapses during the course of the disease in patients was 3.3 (± 1.3) (Table I).

Patients with kidney, liver, endocrine, immunological, inflammatory or infectious disorders were excluded on the basis of history, physical examinations and laboratory evaluations. No patients had received anti-inflammatory, immunosuppressive, immunomodulatory, steroid or hormonal treatment for at least three months before this study-point.

Cerebrospinal fluid (CSF) samples were collected from patients with active MS relapse before the initiation of IVMP therapy (A_0). Venus blood samples were collected from patients in active relapse - before IVMP therapy (B_0), after five days of IVMP therapy (B_5) and in remitting period (B_R). The control group consisted of 10 age-adjusted healthy volunteers whose blood and CSF were being collected for clinical indications.

The study was approved by the scientific ethics committee of the Medical University in Lublin, Poland. All persons gave their informed consent prior to their inclusion in the study.

CSF was concentrated by vacuum centrifugation (JW Electronic, Poland).

Table I. Demographic and clinical characteristics of multiple sclerosis patients (n = 37)

Age (years)	35.2 ± 5.3
Disease duration (years)	6.4 ± 5.1
Number of relapse	3.3 ± 1.3
EDSS before treatment (range)	3.7 (1.0-5.5)
EDSS in the remitting period (range)	2.2 (1.0-4.0)
Gender female/male ratio	20/17

EDSS – Expanded Disability Status Scale (Kurtzke, 1983)

Biochemical investigation

1. To estimate the thiobarbituric acid-reactive material (TBAR) content in CSF or serum of blood we used a spectrophotometric technique of absorption determination modified from Slater and Sawyer [38]. CSF or serum of blood samples were extracted with 20% trichloroacetic acid, and centrifuged at 3000 × g for 10 min. 0.67% thiobarbituric acid (TBA) diluted in buffer (26 mM Tris-HCl, pH 7.0) was added to supernatant. The mixture was heated in a water bath at 100°C for 10 min. After cooling the sample, the absorption level was read spectrophotometrically at the light wave length of 532 nm against a reagent assay. TBAR content was quantified as the malondialdehyde (MDA) extinction molar coefficient E = 1.56 × 105 M^{-1} cm^{-1}, and the result was expressed in MDA nmoles per mg protein.

2. The amount of sulfhydryl (SH) groups, including the estimation of both of total SH groups (t-SH) and of the so-called non-protein soluble SH groups (s-SH) was estimated in CSF and serum. The term non-protein SH groups denotes those originating from SH compounds present in the supernatants obtained after precipitation of proteins with trichloroacetic acid. SH group content was determined by a modified method of Sedlak and Lindsay [36], based on the Ellman spectrophotometric technique using dithionitrobenzoic acid (DTNB). In computations the molar coefficient E = 13 983 M^{-1} cm^{-1} was used. Its value was determined by investigation of cysteine and reduced glutathione standard solutions. Total protein was measured by the method of Lowry *et al*. [20] using bovine serum albumin as standard.

3. Superoxide dismutase (SOD-1) activity were assayed in serum using the methods of Misra and Fridovich [27]. SOD-1 activity was determined at 37°C by recording the increase in absorbance at 480 nm following the autooxidations of adrenaline, inhibited by SOD-1. One unit (U) of this activity is defined as the amount of enzyme inhibiting the adrenaline autooxidation in 50%. Haemoglobin concentration in the haemolysate was estimated after conversion into the cyanmethaemoglobin form using a commercial reagent (Biomed, Lublin, Poland) at 540 nm.

Table II. TBAR content in CSF (group A_0) and in serum of patients with MS pathology at three different time-points, at relapse (group B_0), after 5 days of IVMP (group B_5) and in the remitting period (group B_R), compared with controls

Group	TBAR level nmoles/mg protein	
	(Mean ± SD)	%
Controls *N* = 10	0.3075 ± 0.035	100
Group A_0 *N* = 37	0.5592 ± 0.0767*	181.85
Group	**TBAR level nmoles/mg protein**	
	(Mean ± SD)	**%**
Controls *N* = 10	0.8125 ± 0.0222	100
Group B_0 *N* = 37	1.6646 ± 0.1511*	204.87
Group B_5 *N* = 37	1.4214 ± 0.1353*#	174.94
Group B_R *N* = 37	1.1332 ± 0.1394**#	139.48

** Significantly different from controls, p < 0.001*
*** Significantly different from controls, p < 0.05*
 # Significantly different from B_0 group, P < 0.05

Data analysis

The results were expressed as the mean ± SD. The statistical significance of the differences was determined by analysis of variance (ANOVA) followed by Student's *t* test for paired samples. A value of $p < 0.001$ and $p < 0.05$ was regarded as significant.

Results

In the biochemical investigation of patients with MS, selected parameters (i.e., free radical peroxidation products TBAR, SOD and SH groups content) turned out to significantly differentiate between MS patients and controls. The obtained results are presented separately for each patient group (cf. methods).

The results of TBAR (Table II) suggested, that peroxidation is taking place in MS, as observed in all examined groups, especially the relapse group. We noted that the level of TBAR increased by 81.85% in CSF during relapse compared with controls ($p < 0.05$). In serum of patients in all examined groups, we observed significantly 3 times higher levels of TBAR than in CSF. In serum during relapse, this level was higher by 104.87% ($p < 0.001$) compared with the

control values. After five days of IVMP administration, the levels of TBAR decreased by 30.93% compared with the relapse group, but was still higher by 74.94% than in controls ($p < 0.001$). During the remitting period the level of TBAR decreased in comparison with the relapse and B_5 groups, but was still higher by 39.48% compared with controls' ($p < 0.05$).

In CSF and serum (Table III) we noticed statistically insignificant slight increases in the levels of t-SH groups in MS relapse in comparison with control group levels. After IVMP treatment, the level of t-SH groups in serum decreased compared with controls' levels ($p < 0.05$). During the remitting period the level in serum also decreased in comparison with the level in the control group. The level of s-SH in CSF was higher by 65.71% than in the control group ($p < 0.05$). In serum, the level of s-SH group also increased by 30.66% compared with controls' levels ($p < 0.05$). The treatment prompted an increase in the level of s-SH by 42.79% ($p < 0.001$). In the remitting period the level of s-SH increased by 8.20% compared with the control group ($p < 0.05$), but decreased by 22.46% compared with the level in MS relapse ($p < 0.05$) and by 34.59% compared with the level after five days of treatment ($p < 0.05$). Considering the above described groups, the level of p-SH groups was as follows. In CSF and serum it decreased respectively by 31.72% ($p < 0.001$) and 28.97% ($p < 0.001$) in comparison with the levels in controls. After five days of treatment the level in serum decreased by 58.27% compared with controls' levels ($p < 0.001$). In the remitting period, a slight increase in the level was shown compared with the level in MS relapse and after five days of treatment, but it was still lower by 18.83% in comparison with the level in controls ($p < 0.05$).

The level of SOD-1 (Table IV) in serum during relapse decreased slightly by 4.48% compared with the controls' levels ($p < 0.05$); however, after five days of treatment it increased by 11.34% ($p < 0.05$) and during remission by 9.7% ($p < 0.05$) compared with the controls.

Discussion

MP is used as a standard drug in treating MS relapses. Studies in the literature on the effects of MP

Table III. Thiol groups in CSF at relapse (group A_0) and in serum of patients with MS at three different time-points, at relapse (group B_0), after 5 days of IVMP (group B_5) and in the remitting period (group B_R), compared with controls

Group	Total SH (t-SH) nmoles/mg protein		Soluble SH (s-SH) nmoles/mg protein		Protein SH (p-SH) nmoles/mg protein	
	(Mean ± SD)	%	(Mean ± SD)	%	(Mean ± SD)	%
Controls $N = 10$	58.24 ± 1.04	100	21.0 ± 1.22	100	37.2 ± 1.2	100
Group A_0 $N = 37$	60.24 ± 1.08	103.43	34.8 ± 1.14*	165.71	25.4 ± 1.15**	68.28
Group	**Total SH (t-SH) nmoles/mg protein**		**Soluble SH (s-SH) nmoles/mg protein**		**Protein SH (p-SH) nmoles/mg protein**	
	(Mean ± SD)	**%**	**(Mean ± SD)**	**%**	**(Mean ± SD)**	**%**
Controls $N = 10$	97.14 ± 1.14	100	49.12 ± 0.8	100	48.02 ± 0.84	100
Group B_0 $N = 37$	98.29 ± 1.04	101.18	64.18 ± 2.14*	130.66	34.11 ± 1.18**	71.03
Group B_5 $N = 37$	90.18 ± 1.24*	92.84	70.14 ± 1.82**#	142.79	20.04 ± 1.30**#	41.73
Group B_R $N = 37$	92.18 ± 0.9	94.89	53.15 ± 1.26*#	108.20	38.98 ± 1.16*	81.17

** Significantly different from controls, p < 0.05*
*** Significantly different from controls, p < 0.001*
 # Significantly different from B_0 group, P < 0.05

Table IV. Superoxide dismutase (SOD-1) in serum of patients with MS pathology at three different time-points, at relapse (group B_0), after 5 days of IVMP (group B_5), in the remitting period (group B_R), compared with controls

Group	Superoxide dismutase SOD-1 U/g Hb	
	(Mean ± SD)	%
Controls $N = 10$	2680 ± 1010	100
Group B_0 $N = 37$	2560 ± 1012*	95.52
Group B_5 $N = 37$	2984 ± 94*#	111.34
Group B_R $N = 37$	2940 ± 98*#	109.70

** Significantly different from controls, $p < 0.05$*
*** Significantly different from controls, $p < 0.001$*
 # Significantly different from B_0 group, $P < 0.05$

on the immune system in MS are controversial. Some authors [4,9,23,26] support the view that MP treatment induces a decrease in CD4+ cells. However, there are other reports stating that corticosteroids have no effect on the cell subpopulation [5,8,15]. Reactive oxygen species (ROS) are produced upon interaction of monocytes with brain endothelium, which leads to tight-junction alterations, cytoskeleton rearrangements, loss of blood-brain barrier integrity, and subsequent extravasation of leucocytes into the CNS [44]. Some publications support the view that the generation of ROS enhances in demyelinating diseases [3,21,40,42]. When free radicals concentration exceeds the level that can be handled by defence system, they can cause tissue destruction by disrupting biomembrane structure and functions, SH-containing enzymes, polysaccharide structure and nucleic acid activity [40,43]. The nature and degree of CNS damage can be inferred from alterations in CSF and serum levels of these products [37].

In this study we observed significantly higher CSF and serum levels of TBAR in MS patients during relapse compared with controls, which indicated an increase of peroxidation products in the relapse phase of MS. Our findings were in accordance with results described by Koch *et al*. [16] and Ortiz *et al*. [30]. It was found that TBAR level in the serum decreases after MP treatment and had the lowest value during the remission phase of the disease compared with controls. This indicated an enhanced process of lipid peroxidation in the CNS and probable damage of biomembranes in the brain [17,43]. The lipid peroxidation process intensity was differentiated across the MP treatment; the lowest TBAR level was noted in groups of patients in remission phase and the highest (significantly higher than that in the controls) in patients with relapse of MS.

Protein thiols are important for structure and function of many proteins [29], whereas non-protein thiols are important for the cellular defence system against free radicals [44].

In relapse phase, patient s-SH groups were elevated in CSF and the serum compared with the controls, whereas patient p-SH groups were decreased. After MP treatment, patient s-SH groups significantly increased in serum compared with the controls and patient p-SH groups significantly decreased. SH compounds such as reduced GSH, submit to peroxidation (dehydrogenation) and are transformed into the disulphide compounds (RSSR). Changes in the mutual transformation of SH and RSSR result in a modification of the oxidative processes involving lipid acid dehydrogenase, GSH reductase and thioredoxin systems. In all groups of patients, differences in the decrease of the number of p-SH groups were correlated with an increase in TBAR levels, which suggested that non-protein thiols were in a strategic position against ROS-mediated brain damage [2,19,25,44]. The level of SOD-1 superoxide dismutase increased after MP treatment and this rise was retained during remission.

Our study partially confirmed the results of Naidoo and Knapp [28], who showed higher concentrations of MDA in the serum but did not observed changes of MDA in CSF of patients during remission (we estimated MDA in CSF during relapse). On the other hand Hunter *et al*. [13] demonstrated significant increases in the level of MDA in CSF of patients with MS.

In conclusion, we noticed significant difference between SM patients and control group in peroxidation products. The changes were better seen during relapses of the disease. Following MP treatment the levels of these indicators approached control values especially in the remission phase of the disease. One of oxidative stress exponents is an increase of MDA level in serum – a marker of lipids' peroxidation. This happens both in CSF and the serum during the relapse phase. Also, the changes in thiol groups testi-

fy the beginning of oxidative-reductive reaction in the relapse phase of the disease and the inhibition of free radicals activity after the treatment and in the remission phase. The biggest antioxidative importance is arrogated to SOD-1 which catalyzes dismutation of superoxide anion and, together with catalase and glutathione peroxydase, they perform protective activities against intracellular accumulation of free radicals. Decrease of activity of SOD-1 leads to cell exposure to harmful effect of free radicals. Increase of SOD-1 level after treatment and during remission period testify the protection against free radicals activity. The changes suggest that glucocorticoids protect against free radicals' attacks and cause the stabilization of investigated indicators in the remission period.

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