Journal of Clinical Neuroscience 28 (2016) 38–42

Contents lists available at ScienceDirect

Journal of Clinical Neuroscience

journal homepage: www.elsevier.com/locate/jocn

Clinical Study

Increased S-nitrosothiols are associated with spinal cord injury in multiple sclerosis

Vera Fominykh [a,*], Mikhail V. Onufriev [a], Anna Vorobyeva [b], Lev Brylev [a], Alexander A. Yakovlev [a], Maria N. Zakharova [b], Natalia V. Gulyaeva [a]

[a] Institute of Higher Nervous Activity & Neurophysiology RAS, Department of Functional Biochemistry of the Nervous System, Butlerov Street 5A, Moscow 117485, Russia
[b] Research Center of Neurology Russian Academy of Medical Sciences, Neuroinfection Department, Moscow, Russia

ARTICLE INFO

Article history:
Received 27 October 2014
Accepted 6 September 2015

Keywords:
Multiple sclerosis
Nitric oxide
S-nitrosothiols
Spinal cord

ABSTRACT

Multiple sclerosis (MS) is an immune-mediated disorder associated with inflammation, demyelination and axonal damage. In search of potential biomarkers of spinal cord lesions in MS related to nitric oxide metabolites, we measured total nitrite and nitrate levels, and protein-bound nitrotyrosine and S-nitrosothiol concentrations in the serum of MS patients at different stages of the disease. Sixty-eight patients and 36 healthy volunteers were included in the study. Total nitrite and nitrate levels were augmented in relapsing-remitting MS, while increased S-nitrosothiol concentrations were found both in relapsing-remitting and secondary-progressive MS. Further analysis demonstrated that S-nitrosothiol levels were selectively increased in patients with spinal cord injury. The data suggest that high S-nitrosothiol concentration may be a potential serum biomarker for spinal cord injury in MS.

1. Background

Multiple sclerosis (MS) is an inflammatory demyelinating and neurodegenerative disease affecting the central nervous system and frequently occurring in young adults [1]. Foci of disseminated demyelination are manifested by variable axonal damage and reactive gliosis. Clinically, MS is manifested by episodes of neurological dysfunction, or relapses, related to the site of the lesion. The evolution of the disease is quite variable.

There is abundant evidence that nitric oxide (NO) production is significantly augmented in MS due to inducible nitric oxide-synthase (iNOS) activation (see Smith et al. for review [2]). The involvement of iNOS was confirmed by the data obtained from histopathological studies of lesion sites, analyses of the cerebrospinal fluid, blood and urine of MS patients, as well as from studies on rodents with experimental allergic encephalomyelitis (EAE; an animal model of MS) [3]. Increased production of NO in MS (as well as in some other neurological disorders) results in a generation of reactive nitrogen species interacting with different classes of biomolecules and interfering with their structure and function. This phenomenon, called nitrosative stress [4], has been investigated for several years from the perspective of the deleterious roles of NO and peroxynitrite in demyelinating axonal damage and disruption of the blood–brain barrier [5]. Recent data suggest that most of the cytotoxicity attributed to NO is mediated by peroxynitrite, while NO itself can play immunoregulatory and neuroprotective roles. This may explain why inhibition of iNOS did not provide encouraging results in EAE models [6,7], while endothelial nitric oxide-synthase (eNOS) and iNOS knockout mice eventually demonstrated more severe EAE and delayed recovery as compared to wild type mice [8], thus indicating the ambiguity of NO-mediated mechanisms in the immune response. As a consequence, a dual role of NO in MS has been debated over the last several years in parallel with a discussion on a dual role of neuroinflammation [9–11].

The aim of our study was to assess recognized biomarkers of NO-system activity: protein-bound nitrotyrosine (NTprot), nitrite/nitrate (NOx) and S-nitrosothiol levels (RSNO level; in serum preferably including S-nitrosogluthatione [GSNO] and S-nitrosoalbumine) in the blood at different stages of MS and to determine whether these compounds are associated with different locations of MS lesions in the central nervous system.

2. Subjects and methods

2.1. Patients and control subjects

In this study we examined two groups of patients: patients with MS (n = 56), patients with acute disseminated encephalomyelitis (ADEM; n = 12) and a control group (n = 36).

* Corresponding author. Tel.: +7 495 952 4007; fax: +7 495 334 7020.
E-mail address: hydrohinon@mail.ru (V. Fominykh).

http://dx.doi.org/10.1016/j.jocn.2015.09.017
0967-5868/

Thirty-nine women and 19 men visiting the Research Center of Neurology, Russian Academy of Medical Sciences, were selected for the study and classified as having definite MS according to the criteria of McDonald et al. [12]. The research protocol was approved by the local Ethical Committee. All patients with relapsing-remitting multiple sclerosis (RRMS) were examined at the time of MS attacks. The exclusion criteria were: forms of MS other than RRMS and secondary progressive multiple sclerosis (SPMS); presence of comorbid neurodegenerative, psychiatric or somatic pathology; history of alcohol or drug abuse, head trauma, vascular diseases or seizures; severe signs of depression; and previous immunomodulatory treatment. All patients had aquaporin-4 antibody negative profiles. Neurological assessment, MRI of the brain and spinal cord, and sera examination were performed in all patients. MRI was completed according to the Revised Criteria from the Annual Meeting of the Consortium of MS Centers [13]; MRI of the head and thoracic and cervical sections of the spinal cord was performed for all patients. If clinical symptoms were evident, MRI of the lumbosacral segment was also performed. Neurological impairment was assessed by the Kurtzke Expanded Disability Status Scale (EDSS) and this index was used as an indicator of relapse severity. Twenty-eight patients had been treated with high dose intravenous methylprednisolone during their previous relapse, at a minimum of 3 months before the study commenced. Forty-three patients had MRI lesions in the spinal cord and 47 patients had MRI lesions in the brain.

All patients with MS were subdivided into two subgroups: RRMS and SPMS. The disease activity was classified according to the Lublin and Reingold criteria [14]. The ADEM group consisted of 12 patients. All patients had large multifocal brain MRI lesions but none of them had spinal cord injury. Because the EDSS rating scale does not consider a decline of consciousness which is a crucial criterion for ADEM patients, we did not verify the EDSS score in ADEM patients. Full demographic and clinical characteristics of the patients are presented in Table 1.

Thirty-six healthy subjects were included into the control group (mean age 38.6 ± standard deviation [SD] 8.4 years). There were no significant differences between patients with MS, ADEM and the control group with regard to age and sex ($p > 0.05$).

Table 1
Demographic and clinical characteristics of patients

	RRMS	SPMS	ADEM
Patients	42	14	12
Age at examination, years	32.7 (±7.9)	43 (±8.0)	37.5 (±10.6)
Male/Female	11/31	6/8	5/8
Disease duration	27 months (IQR 2 weeks to 18.1 years)	14.3 years (IQR 4 to 31 years)	3.5 months (IQR 2 weeks to 2.1 years)
Average EDSS score	3.2 (±1.4)	6.1 (±1.6)	–
Time between presenting relapse symptoms and blood sampling, days	25.0 (±32)	–	–
Methylprednisolone therapy in previous relapse, at least 3 months before blood sampling (treated/non-treated)	15/27	13/1	7/5
Spinal cord lesions on MRI (yes/no)	29/13	14/0	0/12
Length of spinal cord lesion (vertebral body units)	1.6 (± 0.8)	2.8 (± 1.2)	0
Brain lesions on MRI (yes/no)	33/9	14/0	12/0
Cerebrospinal fluid cytosis (cell/3 μl)	14.7 (±8)	–	22.1 (±30)
Cerebrospinal fluid protein level (g/l)	0.26 (±0.12)	–	0.39 (±0.2)
Albumin index	5.7 (±2.3)	–	8 (±0.3)

ADEM = acute disseminated encephalomyelitis, EDSS = Expanded Disability Status Scale, IQR = interquartile range, RRMS = relapse-remitting multiple sclerosis, SPMS = secondary progressive multiple sclerosis.
Data are presented as mean ± standard deviation unless otherwise stated.

2.2. Sample collection and materials

Fasted venous blood samples were collected from all patients and control subjects after giving informed consent and before the initiation of treatment. The sera samples were prepared by centrifugation in Clot Activator tubes (Vacutainer; Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for 20 minutes at 1500 g at 4°C. Supernatants were aliquoted in sterile tubes and stored at −70°C. We used bovine serum albumin (MP Biomedicals, Santa Ana, CA, USA), Tween 20, 3-nitro-Ltyrosine (Sigma-Aldrich, St Louis, MO, USA), tetranitromethane, O-orthophenylenediamine dihydrochloride, N-(1-Naphtyl) ethylenediamine dihydrochloride, vanadium chloride(III) and sulphanilamide (Sigma Aldrich), monoclonal antibodies to nitrated bovine serum albumin goat anti-mouse immunoglobulin G-horseradish peroxidase (Cayman Chemical Company, Ann Arbor, MI, USA and Jackson ImmunoResearch, West Grove, PA, USA respectively). For fluorometry we used 4, 5 diaminofluorescein (DAF-2, Cayman Chemical Company).

2.3. Detection of NO metabolites

NOx levels in serum samples were measured using the Griess method [15]. For measurements of NTprot we used an enzyme-linked immunosorbent assay as described in our previous study [16]. Total RSNO levels were assessed using DAF-2 according to Wink et al. [17]. Briefly, 100 μl of samples with 95 μl of DAF-2 solution in 10 mM PBS in deionized water (C = 1.38 μM) had 10 μl of 10 mM $CuSO_4$ solution added. This mixture was incubated for 15 minutes at room temperature. Measurements were performed using a Hitachi F 3000 (Hitachi Aloka, Tokyo, Japan). The slit width was 5 nm for excitation (480 nm) and 10 nm for emission (515 nm). Serial dilutions of GSNO from 62 nM to 4 μM were used for a standard curve construction. GSNO was synthesized from glutathione according to Stamler et al. [18]. DAF-2 and S-nitrodoglutathione solutions were prepared immediately before experiments. Other samples were treated with Amicon Ultra Centrifugal Filters (Merck, Darmstadt, Germany) and analyzed for low-weight and protein-bound RSNO. Sample measurements were performed in duplicate, values for calibration curves in triplicate.

2.4. Statistical analysis

Data analysis was performed using a multiparametric logistic non-linear regression model for NTprot (GraphPad Prism 5; GraphPad Software, San Diego, CA, USA) and a linear model for total NOx and RSNO levels (Excel; Microsoft, Redmond, WA, USA). For statistical analysis we used Statistica 8.0 (StatSoft, Tulsa, OK, USA) and SigmaPlot 11 (Systat, San Jose, CA, USA). Data are expressed as mean ± SD. A non-parametric Kruskal–Wallis test was used for multiple comparisons (significant p value <0.05). For comparisons of patients with controls the non-parametric Mann–Whitney U test with Bonferroni correction was used. Spearman correlations between serum NO metabolites levels and clinical data were calculated and logistic regression was performed to determine significant associations of predictor variables (sex, age, EDSS scores, methylprednisolone treatment, biomarkers levels) with outcomes (phase of disease or spinal cord demyelination). The value of NO metabolites levels as biomarkers for spinal cord injury was assessed by measuring the area under the receiver operating characteristic curve.