Oxidative stress in serum and peripheral blood leukocytes in patients with different disease courses of multiple sclerosis

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Abstract Background The role of oxidative stress in patients with multiple sclerosis (MS) is poorly understood. Objective To investigate oxidative stress in serum and peripheral blood leukocytes in patients with different disease courses of MS. Methods Diene conjugate (DC) levels (a measure of lipid peroxidation), total antioxidative activity (AOA) and total antiradical activity (ARA) were measured in serum and peripheral blood leukocytes from 30 patients with benign relapsing remitting MS (BMS), 27 with secondary progressive MS (SPMS), 29 with primary progressive MS (PPMS), and 30 healthy controls. All MS patients were in a clinically stable phase. Results Serum DC levels were elevated in patients with BMS (p < 0.05), SPMS (p < 0.01) and PPMS (p < 0.001). Serum total AOA and ARA were not different between MS patients and controls. Compared to controls, leukocyte DC levels were not different in each MS subgroup, but total ARA was elevated. There was a strong correlation, both in controls and MS patients, between leukocyte DC levels and leukocyte total ARA (p < 0.0001) and leukocyte total AOA (p < 0.0001). Conclusion Oxidative stress occurs in progressive as well as benign MS. The finding that cells withstand oxidative stress, due to upregulated cellular antioxidant defence mechanisms, suggests that reactive oxygen species (ROS) formation in MS is not necessarily deleterious.

Keywords multiple sclerosis · reactive oxygen species · antioxidant capacity · oxidative stress

Introduction

Reactive oxygen species (ROS) in multiple sclerosis (MS) are generally thought to be derived from activated inflammatory cells, and to play a role in demyelination and axonal damage [15]. Oxidative stress occurs when the production of ROS exceeds their neutralization by antioxidant defense systems. ROS is a collective term for both radical and non-radical but reactive species derived from oxygen, which include superoxide anion (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), hydroxyl radical (OH·). They cause cellular damage because they oxidize cellular components, such as lipids (lipid peroxidation), proteins and DNA.

Only a few studies investigated lipid peroxidation in MS and the results were conflicting. Using infrared microspectroscopy, one study found evidence of oxidative damage of lipids in postmortem MS lesions [25], whereas another study failed to detect such changes [10]. Some studies found increased lipid peroxidation products in plasma of MS patients [26], whereas others did not [16]. Patients with relapsing MS had higher ROS formation than those in remission [31]. One study found
We measured levels of diene conjugates (DC), which are endproducts of lipid peroxidation, and thus serve as a marker of ROS formation, in serum and peripheral blood leukocytes of clinically stable patients with different disease courses of MS. We also quantified total antioxidant activity (AOA; the ability to scavenge H₂O₂) and total antiradical activity (ARA; the ability of free radical scavenging) as measures of antioxidant capacity.

Patients and methods

Patients
The study was approved by the medical ethics committee of the University Medical Center Groningen. All patients gave informed written consent before inclusion into the study. Venous blood samples were obtained between 8 AM and noon through an intravenous cannula in the forearm from 30 healthy controls and 86 patients with definite MS. Exclusion criteria were a relapse or use of corticosteroids within the past 3 months, the presence of infections or fever, and pregnancy. Defined onset symptoms were used to establish the year of disease onset. Of the patients, 30 had relapsing-remitting MS with a relatively benign course (BMS), 27 had secondary progressive MS (SPMS), and 29 had primary progressive MS (PPMS). BMS was defined as an Expanded Disability Status Scale (EDSS) of 3.0 or less despite at least 10 years of disease duration [28]. Patients with SPMS started with a relapsing-remitting course but subsequently accumulated progressive disability. The term PPMS was used for patients whose disease course was progressive from the onset without preceding relapses. Fifteen patients were using interferon β; no other immunomodulatory or immunosuppressing treatment was used. None of the patients or control subjects was following a special diet.

Leukocyte isolation procedure
Erythrocytes were allowed to sediment in 1 ml of a 5% dextran solution (Pharmacia, Uppsala, Sweden) for 90 minutes at room temperature. The leukocyte rich upper layer was removed and the leukocytes were isolated according to the method of Percy and Brady [27]. The final suspension of leukocytes was mixed with 2 ml aqua bidest and the leukocyte lysates were stored at -20°C until used.

Measurement of DC
The content of DC in serum and leukocyte lysates was assessed as described previously [3, 22]. Briefly, 0.5 ml of sample was added to 2 ml of a 1:1 heptane-isopropanol solution. The mixture was cooled until phase separation occurred. 0.5 ml of the heptane phase, which contains the lipid oxidation products, was aspirated and 2 ml of distilled ethanol was added. Absorbance of this solution was measured at 232 nm and DC content was calculated using a standard curve.

Measurement of total AOA
Total AOA of serum and leukocyte lysates was assessed by measuring the chemiluminescent reaction of riboflavin with hydrogen peroxide (H₂O₂) in the presence of ferrous ions as described earlier [6]. In brief, 0.71 ml of 100mM K₂HPO₄ buffer (pH 9.0), 0.04 ml of 10mM riboflavin solution and 0.05 ml of 25mM FeSO₄ solution were mixed in a luminometer cuvette. 0.1 ml of the sample or 0.1 ml of K₂HPO₄ buffer (control) were added to the mixture. The chemiluminescent reaction was started by adding 0.2 ml of 0.1% H₂O₂ solution and luminescence was measured on a luminometer for 2 minutes at room temperature. The magnitude of lightsum inhibition was calculated and expressed in relative units (RU): 1 RU = 1 – (lightsum sample/lightsum control).

Measurement of total ARA
Total ARA of serum and leukocyte lysates was assessed by measuring neutralisation of the stable free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) as described earlier [21]. 0.25 ml of sample was mixed with 0.25 ml of the protein precipitator phosphotungstic acid (10% aqueous solution). The filtrate of this solution was mixed with 1.5 ml of aqua bidest. Control samples contained only 1.5 ml of aqua bidest. 3 ml of a solution of DPPH in methanol with an absorbance at 517 nm of 0.7 to 0.73 was added. 4 ml of toluene was slowly added and the mixture was centrifuged until phase separation occurred. The absorbance of the upper DPPH-containing phase was measured against toluene. ARA was determined by comparing DPPH neutralization of experimental and control samples using a standard curve.

Statistics
The significance of differences between the control group and the different subgroups of MS-patients (BMS, PPMS and SPMS) were assessed with one-way analysis of variance. P-values were corrected for multiple comparisons according to the Bonferroni-method. The Mann-Whitney U-test was used for comparison between patients using or not using interferon β. Correlations were examined using Pearson’s correlation analysis. All statistical analyses were performed with Graphpad Prism for Windows statistical software package version 4.00 (Graphpad Software, San Diego, USA). Statistical significance was taken to be at the 5% level (p < 0.05).

Results
Demographic data of the subjects are given in Table 1. The control subjects and MS population were well matched regarding sex and age distribution. Serum DC was significantly different between the subgroups (p < 0.0006), with higher levels in PPMS (p < 0.001), SPMS (p < 0.01) and BMS (p < 0.05) than in controls (Table 2). Serum DC levels were significantly higher in PPMS patients compared to BMS patients (p < 0.05). Serum total AOA and total ARA in the MS subgroups were not different from controls.

Leukocyte DC levels and total AOA in each MS subgroup were not different from the control subjects (Table 2). Leukocyte total ARA was significantly different between the subgroups (p = 0.0054). Leukocyte total ARA was higher in all 3 MS subgroups compared with controls, and this was significant for BMS (p < 0.01) and SPMS (p < 0.01; Table 2).

Serum DC levels (mean ± SD) were 69.4 ± 12.3 pmol/mg in interferon β treated patients versus 76.1 ± 16.7 pmol/mg in untreated patients (p = 0.6). Leukocyte DC levels, as well as total AOA and total ARA in serum and leukocytes, were also not different be-