Oxidative Stress and Antioxidant Defenses in Plasma of Patients with Behçet's Disease

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Oxidative Stress and Antioxidant Defenses in Plasma of Patients with Behçet's Disease. Tohoku J. Exp. Med., 1995, 176 (4), 239-248 — Plasma myeloperoxidase (MPO) and glutathione peroxidase (GSH-Px) activities, and malondialdehyde (MDA), ceruloplasmin (Cp), transferrin (TF), thiol (SH), selenium (Se) and copper (Cu) levels were determined in patients with Behçet's disease (BD), in order to investigate whether the plasma antioxidant defense system is impaired in BD. When compared to controls, plasma MDA, Cp, Cu levels and also plasma MPO activity were significantly higher in patients, whereas plasma TF, SH and Se levels, and also plasma GSH-Px activity were lower in BD patients than those in controls. In addition, there were significant and positive correlations between MDA-Cp, MDA-Cu, MDA-MPO, MPO-Cp, GSH-Px-Se, Cp-Cu, and TF-SH parameters, but negative correlations between MPO-TF and Cp-TF parameters in BD patients. Based on these findings, it is concluded that plasma antioxidant defense system is insufficient and impaired in BD. ——— Behçet's disease; plasma myeloperoxidase (MPO); glutathione peroxidase (GSH-Px); malondialdehyde (MDA); plasma antioxidants

Behçet's disease (BD) was first described in 1937 by a Turkish dermatologist, Hulusi Behçet, as a triad of uveitis, aphthous lesions of the oral mucosa and genital ulcerations (Behçet 1937). Recent studies have shown that the disease is a multisystem disorder that affects skin, mucous membranes, eyes, joints, central nervous system and blood vessels. Vasculitis is also defined as a major feature of the disease (Jorizzo et al. 1985). The etiology of this unusual disease remains obscure. Although bacterial or viral infections, genetic, environmental and immunologic factors have been implicated in the pathogenesis of the illness, the exact cause is still unknown (Jorizzo et al. 1985; O'Duffy 1990).

On the other hand, investigations have shown that various functions of polymorphonuclear cells (PMN) in peripheral blood, such as chemotaxis, phagocytosis, lysosomal enzyme activities, and also superoxide radical anion (O_2^-) generation, increased in BD. Consequently, these stimulated neutrophil functions

have been considered to be correlated to the pathogenesis of BD (Matsumura and Mizushima 1975; Yamada et al. 1981; Niwa et al. 1982; Fujita et al. 1984). Especially, oxidative species, including O_2^- , have been proposed as possible mediators for tissue damage associated with BD (Yamada et al. 1981; Niwa et al. 1982).

These earlier observations are increasingly supported with recent studies (Niwa and Mizushima 1990; Pronai et al. 1990, 1991). Further, it is observed that there are alterations in activities of enzymes consisting of oxidant/antioxidant system of PMN leucocytes in BD and suggested that increased levels of oxygen free radicals, as a result of these alterations, may play a role in tissue damage in BD (Doğan et al. 1994).

In view of the findings mentioned above, the present study was planned to examine whether the plasma antioxidant defense system is impaired in BD, and if so, whether there is any correlation between the plasma antioxidant defense system and PMN cells. We, therefore, determined myeloperoxidase (MPO; E.C. 1.11.1.7) and glutathione peroxidase (GSH-Px; E.C. 1.11.1.9) activities, and thiol (SH), transferrin (TF) ceruloplasmin (Cp), copper (Cu), selenium (Se) levels along with lipid peroxidation in plasma of patients with BD.

METHODS

Subjects. The present study was performed on patients with BD attending Ophthalmology and Dermatology Departments of Erciyes University, Research Hospital. Twenty-four patients, 20 male and 4 female, were diagnosed as complete type according to the criteria of International Study Group (ISG) (International Study Group for Behçet's Disease 1990). The ages of the patients ranged from 20 up to 40 years (mean age, 28.54 ± 7.70 years) and the duration of the disease from first symptoms was between 1 and 18 years (average 6.04 ± 4.24 years). At the time of the study, patients were in acute stage of the disease with no systemic medication.

Thirty apparently healthy medical students and hospital personnel (25 male and 5 female), age-matched, were also included as controls in the present study.

Biochemical procedure. Heparinized blood obtained from patients and controls was kept at 4°C for 30 min, then centrifuged at $1500 \times g$ for 15 min. Plasma was carefully aspirated off, leaving the buffy coat and packed erythrocytes, and stored at -70°C until analysis. Only plasma SH measurements were carried out on the same day.

SH concentrations in plasma were estimated by the method of Koster et al. (1986) using the thiol-disulphide interchange reaction between 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) and thiols. Plasma malondialdehyde (MDA, a breakdown product of lipid peroxidation) levels were assayed by a spectrophotometric method (Wong et al. 1987) which was partly modified in our laboratory, without the high pressure chromatography separation step. Briefly, after

boiling at 100°C for 60 min, and cooling, *n*-butanol was added into the tubes and mixed vigorously. The butanol phase was separated by centrifugation and absorbance was measured at 532 nm. The CV value of this modified method was found to be 3.2%. Plasma TF levels were determined by immunoturbidimetric assay using a kit from Bayer Diagnostics (Code 6805, Ames, Orcq-Tournai, Belgium). Plasma Cp values were measured by the method based on ceruloplasmin-catalyzed oxidation of *p*-phenylenediamine (Sunderman and Nomoto 1970).

Plasma Cu and Se levels were assayed by atomic absorption spectrometry (Z-8000, Hitachi, Tokyo), with a Zeeman background correction. Flame technique, with standards made in 10% glycerol; and graphite furnace technique, with palladium modifier and pyrolytically coated graphite tubes were used for Cu (Tietz 1986); and Se (Jacobson and Lockitch 1988), respectively.

GSH-Px activity was measured by the coupled assay described by Paglia and Valentine (1967) using H_2O_2 as substrate. The assay kinetics were calculated by using a molar absorptivity of NADPH of $6.22 \times 10^{-3} \text{ M}^{-1} \text{ cm}^{-1}$ at 340 nm. One unit of enzyme activity was defined as the number of μmol of NADPH oxidized per liter of plasma per minute (Paglia and Valentine 1967). MPO activity was determined by a modification of the *o*-dianisidine method (Goto et al. 1992). One unit of MPO was defined as that degrading one μmol of H_2O_2 per min at 25°C. A molar extinction coefficient of 1.13×10^4 of oxidized *o*-dianisidine was used for the calculation (Goto et al. 1992). Enzyme activities, GSH-Px and MPO, were expressed as U/liter.

Statistical analysis. All measurements were performed in duplicate, and values were expressed as the mean $\pm$ s.d. The significance of the differences between means from patients and controls was calculated by the Student's *t*-test. Correlation analyses were also performed between parameters measured in BD patients. The level of significance was accepted as $p < 0.05$.

RESULTS

The data of the present study are presented in Table 1. When compared to controls, there were significant increases in plasma levels of MDA ($p < 0.001$), Cp ($p < 0.001$), Cu ($p < 0.01$), and also in plasma MPO activity ($p < 0.001$) in patients with BD. On the other hand, plasma TF ($p < 0.001$), SH ($p < 0.001$), Se ($p < 0.001$) levels and also GSH-Px activity ($p < 0.001$) were found to be lower in BD patients than those in controls.

When correlation analyses were performed in patient group, there were positive and significant correlations between MDA-Cp ($p < 0.05$), MDA-Cu ($p < 0.05$), MDA-MPO ($p < 0.001$), MPO-Cp ($p < 0.05$), GSH-Px-Se ($p < 0.001$), Cp-Cu ($p < 0.001$) and TF-SH ($p < 0.05$) parameters; but negative and significant correlations between MPO-TF ($p < 0.05$) and Cp-TF ($p < 0.05$) parameters. However, no correlation was observed among the other parameters. Only significant corre-

TABLE 1. *Malondialdehyde, thiol, transferrin, ceruloplasmin, copper and selenium levels, and glutathione peroxidase and myeloperoxidase activities in plasma of patients with Behçet's disease and controls*

Parameters	BD patients (n = 24)	Controls (n = 30)	p Value
Malondialdehyde (MDA; $\mu\text{mol/liter}$)	4.03 ± 0.76	1.96 ± 0.35	<0.001
Thiol (SH; $\mu\text{mol/liter}$)	457.20 ± 66.44	663.52 ± 74.68	<0.001
Transferrin (TF; g/liter)	1.78 ± 0.46	2.74 ± 0.46	<0.001
Ceruloplasmin (Cp; g/liter)	0.48 ± 0.10	0.31 ± 0.06	<0.001
Copper (Cu; mg/liter)	1.28 ± 0.28	1.07 ± 0.16	<0.01
Selenium (Se; $\mu\text{g/liter}$)	131.66 ± 46.96	196.0 ± 46.30	<0.001
Glutathione peroxidase (GSH-Px; U/liter)	179.34 ± 32.96	405.81 ± 60.11	<0.001
Myeloperoxidase (MPO; U/liter)	279.63 ± 48.12	69.80 ± 10.74	<0.001

Values are mean $\pm$ S.D.

n, The number of subjects.

TABLE 2. *Correlation analysis between parameters measured in patients with Behçet's disease*

Parameters	r	y = ax + b	p Value
MDA-Cp	0.500	$2.22x \pm 3.75$	<0.05
MDA-Cu	0.461	$2.46x \pm 1.22$	<0.05
MDA-MPO	0.650	$1.15x \pm 0.01$	<0.001
MPO-Cp	0.495	$0.18x \pm 0.001$	<0.05
MPO-TF	-0.499	$3.13x - 0.004$	<0.05
Cp-Cu	0.868	$0.09x \pm 0.31$	<0.001
Cp-TF	-0.450	$2.77x - 2.06$	<0.05
TF-SH	0.465	$339.78x \pm 66.15$	<0.05
CSH-Px-Se	0.870	$-90.63x \pm 0.87$	<0.001

lations mentioned above are presented in Table 2.

DISCUSSION

With increasing frequency, the human neutrophil is being implicated as a mediator of tissue-destructive events in inflammatory diseases (Fantone and Ward 1985; Weiss 1989). Activated neutrophils produce more reactive oxygen species (ROS) in inflammatory conditions. ROS can react with all biological substances, especially, polyunsaturated fatty acids (Klebanoff 1988); reactions with these cell membrane constituents lead to lipid peroxidation (Fantone and Ward 1985; Weiss 1989). It has also been speculated that excess lipid peroxides formed in tissues are released into the blood plasma (Kasama et al. 1988). In the present study, the enhanced plasma MDA levels, an index of lipid peroxidation, were observed in our

patients. Although we could not find a previous report about lipid peroxidation in BD, it might be expected and explained in various ways. Indeed, functional abnormalities including excess O_2^- generation in the neutrophils of BD patients have been reported (Niwa et al. 1982; Fujita et al. 1984). Hence, activated neutrophils could damage biological targets through lipid peroxidation in BD. In addition, superoxide scavenging activity (SSA) of PMN has been found to be lower in BD patients (Pronai et al. 1991), and also shown that there were strong negative correlations between SSA of PMN and their O_2^- release, and disease activity (Pronai et al. 1991). According to Pronai et al. (1991), the enhanced O_2^- generation by PMN in vivo may be responsible for the decreased SSA of PMN in BD and PMN might be able to release more O_2^- in tissues. In the study performed in our University, decreased MPO, SOD, catalase and GSH-Px activities, and increased NADPH-oxidase activity in PMN cells were observed in BD patients (Doğan et al. 1994). These findings support those of Pronai et al. (1990, 1991) and clearly indicate that there is significant decrease in SSA of PMN due to decreased enzyme activities in the antioxidant system of PMN in BD. Consequently, more increase in O_2^- generation may lead to more lipid peroxidation in tissues in BD.

On the other hand, activated neutrophils can release substantial amounts of the enzyme MPO into the extracellular fluids, together with O_2^- and H_2O_2 release, after respiratory burst (Weiss 1989). MPO can generate extracellular *hypochlorous acid* (HOCl) that rapidly attacks biological molecules and participates in the generation of chloramines (Klebanoff 1988). Each of these compounds found in plasma has been shown to possess significant cytotoxic activity resulting in lipid peroxidation (Fantone and Ward 1985; Weiss 1989). In the present study, we observed a statistically significant increase in MPO activity in plasma of BD patients compared to controls, and also a positive correlation between MPO and MDA levels in patients. According to these findings, excessive release of MPO by PMN into the plasma may occur during the acute stage of the disease. These findings also confirm the studies indicating a decrease in MPO activity of PMN cells obtained from patients with BD (Yamada et al. 1981; Doğan et al. 1994).

Besides decreased SSA of PMN, SSA of plasma was also found to be lower in BD patients and it shows an inverse correlation with disease activity (Pronai and Arimori 1991). In a previous study we have also found decreased total plasma antioxidant activity in patients with BD (Köse et al. 1993). SOD, catalase and GSH-Px, known as the major primary intracellular antioxidant defense, contribute little to regulate the concentration of oxygen metabolites in extracellular fluids. However, primary antioxidant defenses of normal plasma are mainly concerned with the prevention of transitional metal participation in free radical reactions such as lipid peroxidation (Halliwell and Gutteridge 1990a). Therefore, iron-binding and iron-oxidizing proteins, such as TF and Cp, respectively, are suggested as primary antioxidants of human plasma (Gutteridge and Quinlan

1993).

Although several antioxidant mechanisms have been suggested to explain the proposed protective actions of Cp in iron metabolism, the most biologically important function of Cp is its ferroxidase activity. Cp oxidizes Fe^{+2} to Fe^{+3} and facilitates the binding of iron to TF. Thus, ferric ions are tightly bound to and transported by TF (Halliwell and Gutteridge 1990a, b). Therefore, Cp together with TF inhibit iron-ion dependent $\text{OH}^\bullet$ formation from H_2O_2 and lipid peroxidation. In the present study, the plasma level of Cp was found to be higher, but TF lower in BD patients than those in healthy controls. In addition we observed significant positive correlations between MPO-Cp, and MDA-Cp levels, whereas negative correlations between MPO-TF, and Cp-TF levels were found in BD patients. According to our findings, elevated Cp and decreased TF levels may be related to the inflammation associated with the disease. Since, in inflammatory situations, plasma levels of Cp, known as an acute phase reactant, are increased (DiSilvestro 1989; Muntane et al. 1993), whereas TF, known as a negative acute phase reactant (Tietz 1986), is consumed (Ahmadzadeh et al. 1989). Hence, the low TF concentration in BD patients could contribute to a failure in the plasma antioxidant defense. This is further supported with elevated plasma MPO activity, which was found to be correlated positively with Cp and negatively with TF. Since, it is shown that MPO released by stimulated neutrophils may impair the ability of TF to take up the iron released from ferritin (Monteiro and Winterbourn 1988). On the other hand, elevated Cp observed in BD patients may be a protective response to an increase in circulating unbound Fe^{+2} . However, the increase in Cp levels may not be sufficient in protecting tissues from oxidative damage, as seen in the present study. Further, a negative correlation between Cp and TF levels observed in BD patients was an important finding, which shows that incorporation between TF and Cp functions in normal conditions may impair in BD.

It is also shown that inflammation, an acute phase stress, alters Cu metabolism, and Cu levels are elevated in plasma (Di Silvestro 1989). Cu levels presented here are in agreement with the studies reporting high Cu levels in BD (Shimizu et al. 1979; Cengiz and Gürbayrak 1988; Köse et al. 1993; Doğan et al. 1994). In addition, we observed significant positive correlations between Cp-Cu, and MDA-Cu levels in plasma of BD patients. Recently it is shown that oxidants generated from the MPO system may be responsible for oxidative inactivation of the Cu-binding sites of Cp, and possibly because of the release of Cu ions from Cp, lipid peroxidation is enhanced by stimulated neutrophils and ferritin (Abdalla et al. 1992). Although Cu metabolism in BD is not completely understood, it may be thought that high Cu levels may stimulate lipid peroxidation by enhancing more $\text{OH}^\bullet$ formation and contribute to further increase in lipid peroxidation and also tissue damage seen in BD.

It is speculated that plasma SH groups act as an important extracellular

scavenger of peroxides and are also helpful in protecting the surrounding tissues (Hall et al. 1984). Critical SH groups on cell membranes protect proteins against oxidative damage by MPO-derived HOCl (Weiss 1989), while they are consumed. The reduced plasma SH levels have also been attributed to the actions of H_2O_2 (Hall et al. 1984), and transition metal ions (Halliwell and Gutteridge 1990b) promote oxidation of SH groups; further Cp is a specific oxidase for SH containing small molecules (Rae et al. 1986). In the present study, decreased plasma SH levels were observed in BD patients; and there was a positive correlation between SH and TF levels. ROS generated by stimulated neutrophils, and elevated Cp and Cu levels may diminish the amount of free SH groups in plasma of patients with BD, due to the reasons mentioned above. In addition, the decreases in SH along with TF levels clearly indicate that plasma antioxidant defense mechanisms may be both impaired and insufficient in BD.

Fatty acid hydroperoxides, the primary products of enzymatic and nonenzymatic oxygenation of unsaturated fatty acids, intermediates in the arachidonic acid (AA) cascade, react with transition metal complexes and metalloproteins to generate highly toxic free radicals. Excess lipid peroxides formed in tissues are released into the plasma and reduced to the corresponding alcohols by plasma GSH-Px, shown to be different from the intracellular enzyme (Maddipati and Marnett 1987; Kasama et al. 1988). On the other hand, GSH-Px may be inactivated during oxidative stress (Blum and Fridovich 1985); Cu^{+2} (Takahashi et al. 1987) and toxic ligands such as MDA (Arshad et al. 1991) could partially inhibit plasma GSH-Px activity. Significant decreases in plasma GSH-Px activities of patients with BD presented here were also been found in patients with certain skin disorders, including vasculitis (Juhlin et al. 1982). Since, BD is thought to be the result of vasculitis (Jorizzo et al. 1985) and an imbalance between AA metabolites is accepted to be responsible for inflammation and vasculitis, as now seen in BD (Bozkurt et al. 1989); the decrease in plasma GSH-Px may be related to hydroperoxide pathology. Also excess O_2^- generation by PMN cells, and the presence of high MDA and Cu levels seen in BD may be responsible for the decreased GSH-Px levels in plasma of BD patients.

Apart from these, Se deficiency may be a factor for low GSH-Px levels, because, Se, an essential trace element, is an essential component of GSH-Px (Maddipati and Marnett 1987). Se serves to catalyze the reduction of hydroperoxides produced from oxidized species (Lockitch 1989). Se has also shown to have antiproliferative, antiinflammatory, antiviral and immune modulating effects (O'dell et al. 1991). Since inflammation plays a central part in the expression of BD, these Se effects are of interest. Plasma Se levels were found to be lower in BD patients (Delilbasi et al. 1991; Doğan et al. 1993) and suggested that a deficiency in Se impedes the humoral immune response; and it may play an important role in the etiology or prognosis of some inflammatory conditions, and of signs and symptoms of pathological disorders, such as BD (Delilbasi et al. 1991).

In the present study, we confirmed that plasma Se levels in patients with BD are lower than in healthy subjects. Low Se levels may be a sign of depletion of stores or redistribution of total body Se. Therefore, a defective regulation of GSH-Px together with Se could account for some pathological features of BD. The presence of positive correlation between GSH-Px and Se levels observed in our patients may potentiate these suggestions mentioned above.

To our current knowledge, in BD patients, plasma MPO and GSH-Px activities, and MDA, Cp, TF, SH levels were measured for the first time in our University. These findings are of interest and may help the literature related to the pathogenesis and prognosis of BD.

In conclusion, the findings presented here strongly indicate that plasma antioxidant defense mechanisms are insufficient and impaired in BD.

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