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# Lipid Peroxidation and Erythrocyte Antioxidant Enzymes in Patients with Behçet's Disease

KADER KÖSE, CEVAD YAZICI, NİMET ÇAMBAY, ÖZCAN AŞCIOĞLU[1] and PAKİZE DOĞAN

*Departments of Biochemistry and [1]Dermatology, Erciyes University, Faculty of Medicine, 38039 Kayseri, Turkey*

KÖSE, K., YAZICI, C., ÇAMBAY, N., AŞCIOĞLU, Ö. and DOĞAN, P. *Lipid Peroxidation and Erythrocyte Antioxidant Enzymes in Patients with Behçet's Disease.* Tohoku J. Exp. Med., 2002, **197** (1), 9-16 —— In spite of unknown etiology, it is now accepted that reactive oxygen species (ROS) produced by neutrophils may be related to the pathogenesis of Behçet's Disease (BD). The objective was to investigate whether increased production of ROS may affect erythrocyte oxidant/antioxidant system in patients with BD. The levels of malondialdehyde (MDA), one of the end products of lipid peroxidation, in plasma and erythrocyte, and the activities of superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px), antioxidant enzymes, in erythrocyte, also C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR) were measured in 22 patients in active stage of the disease and also in 30 healthy controls. Increased CRP, ESR, and MDA levels in plasma and erythrocyte and increased SOD but decreased GSH-Px activities in erythrocytes were observed in the patients, when compared to the controls. In addition, significantly positive correlations between plasma and erythrocyte MDA levels, and erythrocyte MDA-CRP, MDA-ESR, MDA-SOD, SOD-ESR and SOD-CRP levels, but negative correlation between plasma MDA and erythrocyte GSH-Px, were found in BD patients. It may be suggested that increased production of ROS in BD, as reflected by higher plasma and erythrocyte MDA levels, may impair erythrocyte membrane integrity and also may lead to the alterations in the erythrocyte antioxidant defense system, as reflected by higher SOD and lower GSH-Px activities in erythrocytes. ——— Behçet's disease; lipid peroxidation; glutathione peroxidase; superoxide dismutase; acute phase reactant

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Behçet's disease (BD) was first described by the Turkish dermatologist, Hulusi Behçet (1937), as "recurrent oral aphtous ulcers, genital ulcers, and hypopion-uveits." Nowadays, BD has gained a multisystemic character involving the eyes, joints, central nervous system, vascular

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Address for reprints: Prof. Dr. Kader Köse, Department of Biochemistry, Erciyes University, Faculty of Medicine 38039 Kayseri, Turkey.

e-mail: kkose@erciyes.edu.tr

and pulmonary systems, the alimentary tract and the mucocutaneous areas (Yazıcı et al. 2001). Although the exact patho-mechanism is still unclear (Ghate and Jorizzo 1999), an excessive production of reactive oxygen species (ROS), including superoxide anion ($O_2^-$), by activated neutrophils has been increasingly considered important since it may be related to the pathogenesis of BD (Niwa et al. 1982; Yamada et al. 1986; Pronai et al. 1991; Takeno et al. 1995). Besides the increased ROS production by neutrophils, decreased superoxide scavenging activity (SSA) in both neutrophils (Pronai et al. 1990, 1991) and plasma (Pronai and Arimori 1991) has also been suggested as the factors which may be responsible for the endothelial tissue damage seen in the patients with BD.

The observations from the studies performed at our university, i.e., decreased antioxidant enzyme activities and altered functions in neutrophils (Doğan et al. 1994), an impaired oxidant/antioxidant balance in plasma (Köse et al. 1995), and higher malondialdehyde (MDA) levels in erythrocytes (Köse et al. 1997) of patients with BD, also concluded that increased production of ROS might lead to the tissue damage in BD.

In addition to these previous studies, it was planned in the present study to investigate whether increased ROS production may affect erythrocyte oxidant/antioxidant system in BD patients. Therefore, MDA, accepted as an index of lipid peroxidation resulting from oxidative damage, in plasma and erythrocyte, and also superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) activities, major components of antioxidant defense system, in erythrocytes of patients with BD were measured, and the relations among them were evaluated.

## Materials and Methods

*Subjects*

The study population consisted of 22 patients, 12 men and 10 women, with a mean age of $33 \pm 9$ years (range 20 to 50 years), and the duration of their disease varied from 2 to 15 years (average, $6 \pm 3$ years). The diagnosis of BD was made according to the criteria of the International Study Group (ISG) (International Study Group for Behçet's Disease 1990). Oral ulcers in all patients; genital ulcers in 15 patients (68%); ocular lesions in 14 patients (64%); cutaneous lesions including erythema nodosum and/or papulopustular lesions and/or acneiform nodules in 19 patients (86%); and positive pathergy test in 10 patients (45%) were observed by physicians. None of the patients had cardiac involvement, vascular complications and arthropathy. All patients were in the inactive stage of the disease and none had received any systemic therapy that might affect the results for at least one month preceding the study. Clinical details of the patients with BD were shown in Table 1.

At the time of blood collection the patients were in the acute stage of the disease. Clinical activity was assessed on the basis of the patients having at least three of the ISG criteria; and also C-reactive protein (CRP) titer more than 8 mg/liter (mean, $13 \pm 5$) and an erythrocyte sedimentation rate (ESR) exceeding 15 mm/hour (mean, $26 \pm 15$). At the beginning of the study a full blood count, peripheral blood smears, routine biochemical analyses (blood glucose, urea, creatinine, uric acid, alanine transaminase, aspartate transaminase, alkaline phosphatase, etc) were also performed in all patients and none had any systemic disease.

A group of 30 healthy controls, 17 men and 13 women, aged from 20 to 51 years (mean, $31 \pm 8$ years), were also included in the study. None had any systemic disease and received either mineral or vitamin drugs. The local ethics committee approved the study protocol and all

TABLE 1. *Clinical details of the patients with Behçet's Disease*

| No | Age (years) | Sex (M/F) | Disease duration (years) | Oral ulcers | Genital ulcers | Ocular lesions | Papulo-pustular lesions | Erythema nodosum | Acneiform nodules | Pathergy test |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 34 | F | 11 | + | + | | | + | | + |
| 2 | 20 | F | 6 | + | + | + | | | + | |
| 3 | 21 | M | 9 | + | + | | + | | | + |
| 4 | 40 | F | 4 | + | | | + | + | | + |
| 5 | 27 | F | 3 | + | + | + | | | | |
| 6 | 29 | M | 5 | + | + | | + | + | | |
| 7 | 38 | M | 8 | + | + | | + | | | + |
| 8 | 20 | F | 4 | + | + | + | | | | |
| 9 | 49 | M | 7 | + | + | + | | | | |
| 10 | 49 | M | 3 | + | + | + | + | | | |
| 11 | 34 | M | 5 | + | | + | + | + | | |
| 12 | 30 | M | 7 | + | | + | + | + | + | + |
| 13 | 40 | M | 7 | + | + | | + | | + | + |
| 14 | 32 | F | 6 | + | + | | + | | | + |
| 15 | 23 | M | 4 | + | | + | + | | + | + |
| 16 | 50 | M | 12 | + | | + | + | | | |
| 17 | 33 | F | 4 | + | + | + | | + | | |
| 18 | 33 | M | 5 | + | | + | + | | + | |
| 19 | 30 | F | 4 | + | + | + | | + | + | |
| 20 | 35 | M | 5 | + | + | + | + | | | + |
| 21 | 44 | F | 15 | + | + | | + | | | + |
| 22 | 21 | F | 2 | + | | + | + | | + | |

M, Male; F, Female.

patients and healthy controls signed an informed consent document.

*Analytical procedure*

Heparinized blood obtained from patients and controls were centrifuged at 2000 rpm for 15 minutes at 4°C. After separating plasma, erythrocytes were washed three times with cold isotonic saline and centrifuged. The packed cells, subdivided into aliquots, and also plasma samples were stored at −20°C until analyses.

MDA levels in plasma (Ohkawa et al. 1979) and erythrocyte (Jain 1988) were measured by spectrophotometrical methods, where in MDA couples to thiobarbituric acid to form pink chromogen compound, which has a maximum absorbance at 540 nm. Plasma MDA levels were expressed as micromoles per liter ($\mu$mol MDA/liter) and erythrocyte MDA levels as nanomoles per gram of hemoglobin measured by Drabkin's reagent (nmol MDA/g Hb).

SOD activity of erythrocyte was assayed by its ability to inhibit the reduction of nitroblue tetrazolium (NBT) with xanthine- xanthine oxidase used as superoxide anion generator (Sun et al. 1988). One unit of SOD was defined by the amount of the enzyme required to inhibit the rate of NBT reduction 50% as determined from the standard curve generated with bovine SOD (Sigma Chemical Co., St. Louis, MO, USA). Erythrocyte GSH-Px activity was determined by the coupled assay described by Paglia and Valentine (1967) using $H_2O_2$ as substrate. One unit of GSH-Px calculated by using molar absorbtivity of NADPH of 6200 $M^{-1}$ $cm^{-1}$ at 340 nm, was defined as micromoles

of NADPH oxidized per minute. The activities of SOD and GSH-Px in erythrocyte were expressed as units per gram of Hb (U/g Hb).

*Statistical analysis*

The significance of the mean differences between groups was assessed by the Student's *t*-test. Relationships among parameters were determined by correlation analyses. The *p*-value less than 0.05 was considered to be significant.

## RESULTS

The mean values of parameters measured in BD patients and healthy controls were given in Table 2. MDA levels in plasma and erythrocytes were found to be higher in the patients than those of the controls. The increase in SOD activity, and the decrease in GSH-Px activity in the erythrocytes were observed in the patients when compared to the controls. Similar significant differences were determined in sex-matched comparisons between patients and controls; but there was no statistically significant difference in sex-matched comparisons in both intra-groups (Table 2).

When correlation analyses were performed among parameters in BD patients, erythrocyte MDA was found to be significantly and positively correlated with plasma MDA, SOD, CRP and ESR levels; and there were positive correlations among SOD activity, CRP and ESR levels. There was also significant negative correlation between plasma MDA and erythrocyte GSH-Px

TABLE 2. *MDA levels and SOD, GSH-Px activities in study groups*

| Parameters | Controls | | | Behçet's patients | | |
|---|---|---|---|---|---|---|
| | Men (17) | Women (13) | Total (30) | Men (12) | Women (10) | Total (22) |
| Plasma | | | | | | |
| MDA ($\mu$mol/liter) | 6.9±1.4 | 6.5±0.9 | 6.8±1.2 | 16.6±4.1[b] | 13.4±3.9[b] | 15.2±4.3[a] |
| Erythrocyte | | | | | | |
| MDA (nmol/g Hb) | 13.5±3.2 | 13.2±2.3 | 13.4±2.8 | 42.7±6.4[b] | 41.0±7.1[b] | 41.9±6.6[a] |
| SOD (U/g Hb) | 2 593±606 | 2 408±497 | 2 513±560 | 3 670±916[b] | 3 977±984[b] | 3 810±938[a] |
| GSH-*Px* (U/g Hb) | 31.7±3.5 | 32.1±6.0 | 31.9±4.6 | 24.0±6.1[b] | 26.9±3.7[c] | 25.3±5.2[a] |

The values are expressed as means ±standard deviation (×±S.D.). Subjects in each group are given in parenthesis.

Statistical comparisons: −Between total patients and controls ([a]$p<0.01$).
−Between patients and controls in respect to sex ([b]$p<0.01$; [c]$p<0.05$).

TABLE 3. *The significant correlations among parameters in BD patients (n:22)*

| Parameters | *r* | *y*: $a+bx$ | *p*-value (<) |
|---|---|---|---|
| plsMDA-ercMDA | 0.62 | $27.44+0.95x$ | 0.01 |
| plsMDA-plsGSH-*Px* | −0.48 | $34.32+0.59x$ | 0.05 |
| ercMDA-ercSOD | 0.78 | $-865.83+111.5x$ | 0.01 |
| ercMDA-CRP | 0.59 | $-4.78+0.42x$ | 0.01 |
| ercMDA-ESR | 0.48 | $-19.72+1.087x$ | 0.05 |
| SOD-CRP | 0.64 | $1.07+0.003x$ | 0.01 |
| SOD-ESR | 0.48 | $-3.46+0.007x$ | 0.05 |
| CRP-ESR | 0.74 | $-5.98+2.37x$ | 0.01 |

pls, plasma; erc, erythrocyte.

(Table 3).

## DISCUSSION

ROS produced in excess may cause toxic effects described as oxidative damage on biological molecules, membranes and tissues. Above all, the oxidation of membrane lipids has been implicated as one of the primary events in the oxidative cellular damage (Halliwell 1991). The most common approach to determine the degree of lipid peroxidation induced by ROS is to measure the breakdown products such as MDA (Rumley and Paterson 1998). In the previous studies, both from Turkey (Köse et al. 1995; Örem et al. 1997) and elsewhere (Freitas et al. 1998), plasma MDA levels were found to be higher in Behçet's patients in the active stage of the disease; as well as in the present study. Increased lipid peroxidation as reflected by higher plasma MDA levels may support the concept that ROS may lead to the oxidative damage in BD patients.

It is well known that peroxy radicals produced excessively in the tissues leak into the blood stream and affect the tissues susceptible to peroxidation; such as erythrocytes (Cross et al. 1987). Therefore, the erythrocytes of Behçet's patients should be prone to oxidative reactions depending on an excessive ROS produced, especially by activated neutrophils and also other sources in the active stage of the disease. Indeed, lipid peroxidation characterized by MDA levels in erythrocytes from patients with BD was found to be significantly higher in the present study, as has been shown for the first time by us previously (Köse et al. 1997). It has also been shown that the addition of MDA to human erythrocytes results in a marked increase in membrane rigidity and decrease in cellular deformability (Pfafferott et al. 1982). The finding that the existence of positive significant correlation between plasma and erythrocyte MDA levels in the present study may confirm that increased oxidative reactions may be involved in damaging membrane structure and function, resulted in an impairment in erythrocyte membrane integrity.

On the other hand, antioxidant enzyme-defense mechanisms in the erythrocytes may also be thought to be altered, as seen in neutrophils (Doğan et al. 1994) and plasma (Köse et al. 1995), in BD patients. SOD and GSH-Px are considered to be major determinants of the antioxidant capacity of the erythrocytes (Clemens and Waller 1987). The first natural defense against oxygen cytotoxicity is provided by SOD, which catalyses the conversion of $O_2^-$ into $H_2O_2$ (Halliwell 1991). GSH-Px, a selenoprotein, specific for GSH as a substrate, catalyses the reduction of a range of peroxides to nontoxic alcohols (Clemens and Waller 1987). In the present study erythrocyte SOD activity was found to be higher, whereas GSH-Px lower in the erythrocytes of BD patients.

To our current knowledge, there are only two previous reports showing SOD levels in BD patients. Tüzün et al. (1998) found that BD patients have lower erythrocyte SOD activity, independent from the stage of the disease. Although there was no any explanation why that decreased SOD activity is lower in BD patients, they postulated that decreased SOD activity leading to the increased ROS production might play an important role in the pathogenesis of BD, in their study (Tüzün et al. 1998). However, Wang et al. (1997) reported that serum SOD activity was markedly increased in BD patients, especially in active BD, along with elevated serum levels of interleukin-8 (IL-8) levels, a neutrophil activator, known to have proinflammatory properties.

In the literature, the results of routine investigations are often within normal range in BD. During an obvious flare of the disease, acute-phase reactants such as CRP and ESR may be moderately elevated (Russell et al. 2001). All of these were confirmed by the findings of the present study. It has also been shown that the SSA of both neutrophils (Pronai et al. 1990, 1991) and plasma (Pronai and

Arimori 1991) in BD are negatively correlated with ESR and CRP levels, indicating that a decrease in SSA inversely correlates with the severity and activity of inflammation. On the other hand, many studies have shown increased SOD activity in inflammatory diseases, including rheumatoid arthritis (RA). SOD activity in synovial fluid of patients with RA was found to be higher and significantly correlated with CRP and the severity of the disease (Igari et al. 1982). In addition, proinflammatory cytokines and mediators have been shown to be elevated in both lesional fluids and serum of BD patients (Mege et al. 1993), and cytokines are known to induce the production of SOD, probably through generation of ROS, in other word, ROS generated by cytokines are known to result in up regulation of this enzyme (Perera et al. 1995). Therefore, the suggestion of Wang et al. (1997) that IL-8 may induce the synthesis of SOD through ROS production or SOD activity may increase in response to ROS produced in excessive, in active BD, may be a great deal of importance.

In the present study, we have shown for the first time, significantly positive correlations between MDA-CRP, MDA-ESR, MDA-SOD, SOD-ESR and SOD-CRP levels in BD patients. Although we do not know the underlying mechanism for the increased SOD activity in these patients, it may, together with ROS, CRP and ESR, be responsible for the manifestation of the acute inflammatory stage of BD.

It is well known that the polymerization of membrane lipids and proteins contributes to the events leading to the removal from circulation of either aging cells or cells exposed to peroxidation-initiating agents (Pfafferott et al. 1982). In addition, BD patients do not usually become anemic, in contrast to many other chronic inflammatory diseases (Russel et al. 2001). Further, younger erythrocytes have also been shown to have higher SOD activity rather than older erythrocytes in piglets (Snityns'kyi et al. 1996). When taken together into consideration, it may be postulated that the rate of erythrocyte turnover may be higher in BD, and therefore the increased SOD activity might reflect the appearance of young erythrocytes having high levels of SOD.

In the report of Tüzün et al. (1998), only one study found in the literature related to the erythrocyte GSH-Px activity in BD, it has been shown that there was no any change in GSH-Px activity in erythrocytes of Behçet's patients, in active or inactive stage. On the other hand, GSH-Px activities were found to be lower in erythrocytes in the present study, and also in both neutrophils (Doğan et al. 1994) and plasma, together with low selenium and thiol levels (Köse et al. 1995), in our previous reports. It has also been shown that GSH-Px activity may be inactivated in oxidative stress conditions, and superoxide anion itself can inhibit peroxidase function (Blum and Fridovich 1985), and toxic ligands such as MDA could partially inhibit GSH-Px activity (Arshad et al. 1991). In addition, the significant negative correlation between plasma MDA and GSH-Px activity was observed in the present study. Therefore, it may be thought that erythrocyte GSH-Px activity may decrease in Behçet's patients in the active stage due to the excess ROS generation and/or higher plasma and erythrocyte MDA levels. On the other hand peroxidase activity depends on the availability of selenium as an essential component and also GSH as a co-substrate (Clemens and Waller 1987; Halliwell 1991); but selenium (Delilbaşı et al. 1991; Köse et al. 1995) and thiol levels (Köse et al. 1995), have been reported to be lower in BD patients. All of these could be considered among possible factors involved in decreased GSH-Px activity in BD patient.

In conclusion, it may be suggested that increased production of ROS in BD, as reflected by higher plasma and erythrocyte MDA levels, may impair erythrocyte membrane integrity and also may lead to the alterations in erythrocyte antioxidant defense system, as reflected

by higher SOD and lower GSH-Px activities in erythrocytes.

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