In Vitro Antioxidant Effect of Ginkgo biloba Extract (EGb 761) on Lipoperoxidation Induced by Hydrogen Peroxide in Erythrocytes of Behçet’s Patients

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ABSTRACT—Excessive superoxide radical production and an impaired antioxidant mechanism in both the neutrophils and plasma of patients with Behçet’s disease (BD) have been reported. To provide clinical support for the earlier data, erythrocyte membrane integrity was investigated by measuring malondialdehyde (MDA, a marker of lipid peroxidation) levels in the erythrocytes of BD patients. In addition, the antioxidant effect of Ginkgo biloba extract (EGb 761) at 25 and 250 µg/ml concentrations on lipoperoxidation induced by hydrogen peroxide (H₂O₂) in erythrocyte obtained from BD patients was examined in in vitro conditions. When compared to healthy controls, basal erythrocyte MDA levels were found to be higher in BD patients. In the in vitro study, there was also a significant increase in H₂O₂-induced MDA production in the medium containing no EGb 761 in the patient group, whereas significant decreases in MDA levels were observed in the mediums containing EGb 761 both in the patient and control groups. The decrease in MDA production was found to be related to EGb 761 concentration. These data indicate that an oxidative damage is present in erythrocytes obtained from Behçet’s patients, and EGb 761, which may strengthen the antioxidant defense system, may contribute to the treatment of BD.

Keywords: Behçet’s disease, Ginkgo biloba extract (EGb 761), Lipid peroxidation, Malondialdehyde, Erythrocyte

Behçet’s disease (BD) is a multisystem disorder which was first described in 1937 by a Turkish dermatologist, Hulusi Behçet, as a triad of aphthous lesions of the oral mucosa, genital ulcerations and hypopyon iritis (1). Besides the original triple-symptom complex, BD may also include ophthalmic, mucocutaneous, neurologic, cardiovascular, pulmonary, gastrointestinal, urogenital and musculoskeletal manifestations (2). The exact etiology of BD is still unknown, but bacterial or viral infections, genetic and environmental factors, toxic chemicals, and autoimmune mechanisms have been implicated in the pathogenesis of BD, and vasculitis has been accepted to have a role in the basis of this disease (3).

Although BD has been reported throughout the world, including North America and England, the syndrome appears to be most common in the Middle Eastern Mediterranean countries and Japan (3, 4). Since BD is seen mostly in young people and many lead to worse clinical symptoms such as blindness (5), the treatment of patients are increasingly considered important.

There is no certain treatment model for the disease, but treatment models for preventing the enflammatuar cell accumulation by decreasing the activities or amounts of these cells, especially lymphocytes and plasma cells, which play a role in the pathogenesis of mucosa, synovia and vasculitis, are primarily considered (6). Up to now, colchicine, interferon, immunosuppressive drugs, cytotoxic agents such as azathioprine, antibiotics such as cyclosporin and frequently corticosteroids have been used as conventional therapy; and many studies assessing the effectiveness of these agents have been published for each treatment model (6–9).

On the other hand, it has been suggested that vascular and endothelial tissue damage seen in BD is dependent on elevated reactive oxygen species (ROS), including superoxide radical anion (O₂⁻) generated by activated neutrophils from BD patients (10, 11). Hence, it may be considered that pharmacological agents that have superoxide
scavenging activity (SSA) may contribute to the treatment of BD.

Recently, in vitro and in vivo experiments have indicated that *Ginkgo biloba* extract (EGb 761) is one of the pharmacological agents that have SSA (12). Our previous studies demonstrated the antioxidant potential of EGb 761 (13, 14).

The first part of the present study was designed to examine the degree of lipid peroxidation, reflected as malondialdehyde (MDA) levels, in erythrocytes of BD patients. In the second part of the study, the antioxidant effect of EGb 761 on lipoperoxidation induced by hydrogen peroxide (H$_2$O$_2$) was investigated in erythrocytes obtained from Behcet's patients. Our results are discussed in relation to the usage of EGb 761 for a treatment support in this disease.

MATERIALS AND METHODS

Subjects

The study included 20 male patients with BD attending the Dermatology Department of Erciyes University, Research Hospital. The patients were diagnosed as the complete type according to the criteria of the International Study Group (ISG) (15). Their ages ranged from 20 to 42 years (mean, 28.35 ± 7.32 years), and the duration of the disease from first symptoms was between 1 and 18 years (average, 6.0 ± 4.19 years). At the time of the study, patients were in the acute stage of the disease with no systemic medication.

A total of 20 male volunteers (aged 18 to 44 years, mean age, 27.90 ± 8.06), without clinical or laboratory evidence of any disease were also included in the study, as a healthy control group.

Informed consent for entry into the study was obtained from all patients and volunteers.

Materials

*Ginkgo biloba* extract, EGb 761, standardized at 9.6 mg ginkgo flavoglycosides per ml, was generously provided by Wilmar Schwabe Pharmaceutical Industry (Karlsruhe, Germany). Other reagents were purchased from Sigma Chemical Co. (St. Louis, MO, USA) or from Merck (Darmstadt, Germany). All chemicals were of the highest purity grade available.

Methods

Heparinized blood obtained from patients and controls was centrifuged at 1500 × g for 15 min at 4 °C. Plasma and buffy coat were discarded, and the erythrocyte pellet was washed three times with ice-cold 0.015 M phosphate-buffered saline (PBS), pH 7.4. The packed cells were then diluted with 0.015 M PBS containing 10$^{-3}$ M sodium azide to the original blood volume. After measuring hemoglobin concentrations with Drabkin's reagent, the susceptibility of erythrocytes to lipid peroxidation was determined immediately by the method based on measuring the concentration of the pink chromogen compound that forms when MDA couples to thiobarbituric acid (16). Basal MDA levels of groups were expressed in nmol MDA per gram of hemoglobin (nmol MDA/g Hb).

The remaining erythrocyte suspensions were used for in vitro experiments. Hemoglobin concentrations of samples were adjusted to a final incubating concentration of 3.75 mg/ml with PBS-azide. *Ginkgo biloba* extract was also diluted with PBS-azide to obtain suitable concentrations.

A 1-ml aliquot of EGb 761 was transferred to each of 2 incubation tubes containing 5 ml of diluted erythrocyte suspensions to give final concentrations of 25 and 250 µg of EGb 761 per ml, respectively. Their blank tubes were prepared by mixing 5 ml of PBS-azide and 1 ml of EGb 761 corresponding to 25 µg/ml or 250 µg/ml concentrations. The mixture of 5 ml of diluted erythrocyte suspension and 1 ml of PBS-azide was used as the control and only PBS-azide as the blank.

All the mixtures were pre-incubated at 37°C for 1 hr. Then, cell suspensions were exposed to H$_2$O$_2$ by the addition of 5 ml of 20 mM H$_2$O$_2$ and then incubated at 37°C for a further 1 hr. MDA produced as the result of induced lipid peroxidation was measured by the method of Stocks and Dormandy (17). The MDA levels were expressed as described above.

The preparations and incubations of all mixtures were carried out simultaneously for each BD patient and volunteer, in duplicate.

Statistical analyses

Student's $t$-test was used for statistical comparison of the data from basal erythrocyte MDA levels of patients and healthy controls. The correlation coefficient was determined by linear regression analysis performed between erythrocyte MDA levels and the age of patients, and also disease duration. The significance of the experimental data was determined by analysis of variance (ANOVA). Differences between means were then analyzed by Scheffe's procedure (post-ANOVA test) (18). Values of $P < 0.05$ were considered significant.

RESULTS

Basal erythrocyte MDA levels of BD patients and healthy controls are shown in Table 1. MDA levels were found to be higher in erythrocytes of patients than those of controls ($P < 0.001$). Positive and statistically significant correlations were observed between erythrocyte MDA levels and the ages of the patients ($r$: 0.830,
Table 1. Basal malondialdehyde (MDA) levels in erythrocytes of patients with Behçet's disease (BD) and in healthy controls

| Healthy controls | Behçet's patients | P value |
|------------------|-------------------|--------|
| MDA (nmol/g Hb)  | 6.13±0.91         | 12.09±1.88 | <0.001 |

MDA values are means ±S.D. for 20 subjects.

P < 0.001) (Fig. 1) and also between MDA and disease duration (r: 0.808, P < 0.001) (Fig. 2).

The effects of EGb 761, at low (25 μg/ml) and high (250 μg/ml) concentrations, on peroxide-induced MDA production in both BD patients and healthy controls are shown in Table 2. When compared with control tubes containing no EGb 761 (MDA production 100%), 25 μg/ml of EGb 761 inhibited MDA production by 15.31% and 23.20% and 250 μg/ml of EGb 761 inhibited it by 55.96% and 52.84% in both healthy controls and patients, respectively. The inhibitory effect of EGb 761 on MDA production in the control and patient groups was dose-dependently increased (P < 0.001).

There was a statistically significant increase in MDA formation in the patient group with no EGb 761 as compared to the control (P < 0.01). In addition, the antioxidant effect of EGb 761 at both concentrations was significantly higher in healthy controls than in that of patients (P < 0.05 and P < 0.01, respectively) (Table 2).

Fig. 1. Relationship between erythrocyte MDA and the ages of Behçet's patients.

![Graph showing relationship between age and MDA](image1)

y = 3.22x - 10.576  
r = 0.830

Fig. 2. Relationship between erythrocyte MDA and the disease duration of Behçet's patients.

![Graph showing relationship between disease duration and MDA](image2)

y = 1.795x - 15.696  
r = 0.808
Table 2. The Effect of Egb 761 on peroxide-induced erythrocyte lipoperoxidation, as indicated by MDA production, in healthy controls and in Behçet’s patients

| Concentration of Egb 761 | Healthy controls | Behçet’s patients |
|-------------------------|-----------------|------------------|
|                         | MDA production  | Inhibition compared with control (%) | MDA production  | Inhibition compared with control (%) |
|                         | (nmol MDA/g Hb)*|                                 | (nmol MDA/g Hb)*|                                 |
| Control (FBS-azid)      | 740.06 ± 39.41  | 0.00             | 893.22 ± 69.37**| 0.00             |
| 25 μg/ml                | 626.75 ± 31.76**| 15.31            | 685.99 ± 54.91*†| 23.20            |
| 250 μg/ml               | 325.91 ± 45.13***,**| 55.96            | 421.20 ± 38.34***,**| 52.84            |

*Values are means ± S.D. for 20 experiments. Significances: Control values compared between healthy controls and Behçet’s patients (**P < 0.01), Egb 761 concentrations compared with the control values in both group (**P < 0.001, **P < 0.01), Egb 761 concentrations compared between healthy controls and Behçet’s patients (P < 0.05, †P < 0.01), Egb 761 concentrations compared with each other in both group (***P < 0.001).

**DISCUSSION**

Behçet’s syndrome is a multisystem disorder accompanied by a very complex pathology. Although the role of ROS in BD is not definitely demonstrated, there is some evidence that functional abnormalities are present in the neutrophils of patients with BD such as chemotaxis and phagocytosis (19); and also stimulated neutrophils from BD patients generate high levels of ROS, resulting in endothelial tissue damage (10, 20). Furthermore, decreased SSA both in polymorphonuclear (PMN) cells (11, 21) and plasma (22) has been found in Behçet’s patients. It was also shown that there is a negative correlation between the SSA of PMN cells and their O₂ release (11), and the SSA is inversely correlated with the disease activity (11, 22). In view of these findings, it has been suggested that the enhanced O₂ generation by PMN may be responsible for the decreased SSA of PMN in BD, and PMN might be able to release more O₂ in tissues (11, 21, 22). These findings have been supported by the studies performed at our University (23, 24). Alterations in enzyme activities making up the oxidant/antioxidant system of PMN leukocytes, decreased superoxide dismutase (SOD), myeloperoxidase (MPO), catalase and glutathione peroxidase (GPx) activities and increased NADPH-oxidase activity, were observed in BD patients (23). Behçet’s patients exhibited increases in plasma MDA, ceruloplasmin, copper levels and MPO activity, an enzyme specific to the neutrophils, but had decreases in plasma transferrin, thiol, selenium levels and GPx activity (24); these observations lead to the conclusion that there is a significant decrease in the SSA of PMN due to decreased enzyme activities in the antioxidant system of PMN (23), and also the plasma antioxidant system is insufficient and impaired in BD, as reflected by increased plasma MPO activity and MDA levels showing the existence of neutrophil activation that may lead to the lipid peroxidation (24). Consequently, a greater increase in O₂ generation may lead to more lipid peroxidation in tissues in BD.

The consequences of increased plasma lipid peroxide levels are mostly observed in susceptible target tissues. Erythrocytes are an early model for studies of oxidative stress (25); furthermore, abnormal susceptibility of erythrocyte membrane lipids to peroxidation is believed to reflect a similar abnormality in other organs and tissues (26). Although erythrocytes should be prone to oxidative reactions because of relatively high oxygen tensions, the presence of Hb, and plasma membrane rich in polyunsaturated lipids (27), the capacity of normal erythrocytes to metabolize extracellular ROS (28) and also H₂O₂ produced by activated neutrophils (29) have been demonstrated because these cells are rich in antioxidant systems that are enzymatic and/or nonenzymatic (27).

To our current knowledge, there has been no previous report on lipid peroxidation in erythrocytes of Behçet’s patients. The present study shows, for the first time, a statistically significant increase in erythrocyte lipid peroxidation characterized by elevated MDA levels in Behçet’s patients, depending on the ages of the patients and also disease duration. These results suggest that erythrocyte membrane integrity is impaired in these patients and lipid peroxides are involved in damaging erythrocyte membrane structure and function, thus promoting the progression of BD. Furthermore, altered antioxidant defense mechanisms in the erythrocytes, due to decreased erythrocyte SSA, may be one of the important factors that cause this peroxidation, as seen in PMN cells (23) and plasma (24) which have decreased SSA, in BD.

For this reason, the usage of pharmacological agents that have SSA in the treatment of Behçet’s patients, to provide a clinical contribution to conventional therapy, is increasingly considered important. Indeed, the topical application of SOD cream has been reported to be useful for the treatment of BD (30). BG-104, a compound of Chinese herbs, has also been reported to enhance SSA in BD (22, 31).
Ginkgo biloba extract is one of the pharmacological agents proposed as a free radical scavenger in the last decade. Extracts obtained from the green leaves of the Ginkgo biloba tree, introduced in the first Chinese pharmacopoeia about 5000 years ago, have been used therapeutically for centuries and in conventional medical practice since 1965 (12). Standardized Ginkgo biloba extract, designated EGb 761, contains ginkgoflavone glycosides (24%, w/w) and terpenoids (ginkgolides A, B, C and bilobalide; 6%, w/w) (32, 33). These active constituents of EGb 761 contribute in varying degrees to the therapeutic actions of the extract (12).

EGb 761 has also been shown to have antioxidant activity. Studies in humans showed that cyclosporin A-induced lipid peroxidation in liver microsomes (34) and the release of ROS (O2·-, HO, OH-) during the stimulation of neutrophils by a soluble agonist (35) are prevented by EGb 761 in a concentration-dependent manner. In a previous study, we also observed that the antioxidant potential of EGb 761 on hydrogen peroxide induced lipoperoxidation in healthy human erythrocyte membranes is increased with increasing EGb 761 dose and with incubation time, under in vitro conditions (13). Furthermore, when the antioxidant effect of EGb 761 is compared with those of water-soluble and lipid-soluble antioxidants under the same in vitro system, EGb 761 has been found to be more effective than ascorbic acid, glutathione and uric acid and as effective as a-tocopherol and retinol acetate (14).

In the present study, it is notable that MDA production induced by hydrogen peroxide is higher in Behçet's patients than healthy controls, whereas the inhibition rates of MDA production by EGb 761 at high concentration is similar in erythrocytes from both BD patients and healthy controls. The higher MDA production followed by H2O2 induction in BD patients may reflect increased ROS production, but a decreased antioxidant mechanism in these patients and, consequently, damage in the membrane integrity of erythrocytes.

The study of Robak and Gryglewski (36) may explain the mechanism underlying the antioxidant effect of EGb 761. According to these authors, the antioxidant properties of flavonoids involve mainly scavenging of O2·-, whereas nonflavonoid antioxidants act on other links of free radical chain reactions, most likely by scavenging OH radicals (36). Hence it may be assumed that the antioxidant action of EGb 761 results from the flavonoid glycosides which remove O2·- and the terpenoids which scavenge OH radicals. In addition, a concentration-dependent SOD activity of EGb 761 has also been described (37, 38). SOD might contribute to the antioxidant activity of EGb 761, presumably by scavenging O2·-.

In addition, the membrane-stabilizing action of EGb 761 has also been demonstrated; it decreases the osmotic fragility of rat erythrocytes and penetrates into the membrane phospholipid domain because it has both hydrophilic and lipophilic characteristics (12).

Those findings may explain the present results that the inhibitory effect of EGb 761 on induced MDA production depended on concentration in the erythrocytes of both Behçet's patients and healthy controls. In conclusion, the present data indicate that the increase in erythrocyte MDA levels might be related to the oxidative damage, supporting previous studies showing excessive ROS production and also impaired antioxidant mechanism in BD. In addition to the conventional treatment of the disease, we believe that the usage of antioxidant drugs like EGb 761, which will strengthen the antioxidant defense system of an organism, may contribute greatly to clinical improvement in BD.

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