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CLINICAL INVESTIGATION

The Status of Oxidants and Antioxidants in the Neutrophils of Patients with Recurrent Aphthous Stomatitis

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Abstract: Recurrent aphthous stomatitis (RAS) is an inflammatory condition of unknown etiology. The exact role of the leukocytes in the pathogenesis of RAS is not known. The aim of this study was to evaluate the status of the oxidative system in neutrophils of RAS patients.

A total of 26 patients and 22 sex and age matched healthy control subjects were analyzed by measuring intracellular oxidant/antioxidant enzymes and related parameters; myeloperoxidase (MPO), xanthine oxidase (XO), catalase (CAT), superoxide dismutase (SOD), adenosine deaminase (AD), malondialdehyde (MDA) and nitric oxide (NO).

Statistically significant increases in the activities of SOD, CAT and levels of MDA, NO were detected in the neutrophils of patients. There was no significant difference in MPO, AD and XO activities of neutrophils.

Although the functions of neutrophils were normal, there may be an oxidative stress affecting neutrophils in RAS.

Key Words: Recurrent aphthous stomatitis, neutrophils, oxidative system

Introduction

Recurrent aphthous stomatitis (RAS) is one of the most common oral mucosal diseases. It has three clinical variants, of a minor, major and herpetiform RAS. Minor RAS is characterized by painful, round or oval shallow ulcers, usually less than 5 mm in diameter. A gray-white pseudomembrane is surrounded by a thin erythematous halo. Minor RAS is the most common variant and constitutes about 80% of RAS cases (1, 2). Major RAS is an uncommon and severe form with ulcers exceeding 1 cm in diameter. Ulcers may persist for up to 6 weeks or more and often heal with scarring (1-3). Herpetiform RAS is the least common type. It is characterized by multiple crops of small, painful ulcers of the oral cavity. Furthermore 100 ulcers may be present at a given time and produce large ulcers with irregular border (1, 3).

Although RAS has been the subject of considerable studies, the etiology and pathogenesis of the disease have

not yet been completely explained (1, 2, 4, 5). Local and systemic conditions, genetic, immunological and infectious factors have been identified as potential etiological agents. The free radical metabolism in the erythrocyte and serum of RAS patients has been investigated in two recent studies (6, 7). Also there are a few studies showing the active role of white blood cells in RAS pathogenesis (8, 9). Although the chemotactic function of neutrophils is normal in RAS (10), their marked concentrations at the ulcer area suggest that they may play an active role in the pathogenesis. However, the exact role of neutrophils in the pathogenesis or healing of RAS is still unknown.

The aim of our study was to evaluate the change in the oxidative system in neutrophils of RAS patients by measuring intracellular oxidant/antioxidant enzymes and related parameters; myeloperoxidase (MPO), xanthine oxidase (XO), catalase (CAT), superoxide dismutase

(SOD), adenosine deaminase (AD), malondialdehyde (MDA) and nitric oxide (NO).

Materials and Methods

Subjects

The study was conducted at Zonguldak Karaelmas University Medical Faculty, Departments of Dermatology and Biochemistry. A total of 26 (14 male and 12 female) patients with minor RAS which recurred not less than four times a year, and had a history of at least one year, were included in the study. The patients were otherwise healthy. Also, they had not been under any systemic or topical therapeutic regimen at least for the last 2 months. All patients having RAS had more than one ulcer present for not more than 48 h at the time of analysis. Twenty two (12 male and 10 female) age and sex matched healthy volunteers with no a history of RAS were selected to form the control group.

The patients and controls with a history of smoking and alcohol habits, systemic disorders, or who were diagnosed to have Behçet's disease according to the diagnostic criteria of the International Behçet Committee were excluded from the study.

Methods

Preparation of peripheral blood leukocytes

A total of 10 ml of venous blood was with drawn from each patient after overnight fasting. Samples were transferred into plastic tubes containing 20mg solid EDTA for the separation of leukocytes by a dextran sedimentation method. After sedimentation, the remaining red cells in the leukocyte fraction were removed by hypotonic shock using chilled water in 0.6mol/L KCl. This separation generally contained 90% neutrophils, whereas the remainder of the cells consisted of monocytes, lymphocytes and eosinophilis. Leukocytes were lysed by repeated thawing and freezing after addition of 0.2% Triton X. After this, supernatants were examined by light microscopy and no leukocytes were found (11).

MDA determination: The MDA level of neutrophils was determined by Draper's method. The levels were measured as thiobarbituric acid reactive substance (TBARS). The principle of the test was based on the formation of a coloured complex at 532 nm with maximum absorbance (12). This complex is a result of the

reactions of TBA and MDA, the last product of fatty acid peroxidation. Levels were expressed as nanomoles (nmol) per gram protein.

XO activity determination: Xanthine oxidase activity was assayed spectrophotometrically at 293 nm and 37°C with xanthine as substrate (13). The formation of uric acid from xanthine results in an increase in absorbency. One unit of activity was defined as 1 µmol of uric acid formed per minute at 37°C, pH 7.5, and expressed in units per gram protein.

NO determination: As NO measurement is very difficult in biological specimens, tissue nitrite (NO_2^-) and nitrate (NO_3^-) were estimated as an index of NO production. The method was based on the Griess reaction (14). Samples were initially deproteinized with Somogyi reagent. Total nitrite (nitrite+nitrate) was measured after conversion of nitrate to nitrite by coppORIZED cadmium granules by a spectrophotometer at 545 nm (Ultraspec Plus, Pharmacia LKB Biochrom Ltd, England). A standard curve was established with a set of serial dilutions (10^{-8} - 10^{-3} mol/L) of sodium nitrite. Linear regression was done by using the peak area from the nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as nmol per gram protein.

SOD activity determination: Total (Cu-Zn and Mn) SOD (EC 1.15.1.1) activity was determined according to the method of Sun et al (15). The principle of the method is based on the inhibition of NBT reduction by the xanthine-xanthine oxidase system as a superoxide generator. Activity was assessed in the ethanol phase of the lyzate after 1.0 ml ethanol/chloroform mixture (5/3, v/v) was added to the same volume of sample and centrifuged. One unit of SOD was defined as the enzyme amount causing a 50% inhibition in the NBT reduction rate. SOD activity was also expressed as Units per milligram protein (U/mg protein)

Adenosine deaminase activity determination: AD activities were estimated spectrophotometrically by the method of Giusti, which is based on the direct measurements of the formation of ammonia, produced when AD acts in excess of adenosine (16). Results were expressed as units per gram protein (U/g protein).

CAT activity determination: CAT activity was determined according to Aebi's method (17). The principle of the method is based on the determination of

the rate constant (s^{-1} , k) or the H_2O_2 decomposition rate at 240 nm. Results were expressed as k (rate constant) per gram protein

MPO activity determination: MPO (EC 1.11.1.7) activity was determined using a 4-aminoantipyrine/phenol solution as the substrate for MPO-mediated oxidation by H_2O_2 , and changes in absorbance at 510 nm (A_{510}) were recorded (9). One unit of MPO activity was defined as that which degraded 1 $\mu\text{mol } H_2O_2/\text{min}$ at 25°C. Results were presented as milli units per gram tissue protein.

Protein determination: Protein measurements were made according to the method of Lowry et al (18). All samples were assayed twice.

Statistics: All values were expressed as mean \pm standard deviation. SPSS for Windows 11.0 was used for statistical analysis. Student's *t*-test was used to estimate the significance between parameters. The differences were considered to be significant when the probability was less than 0.05.

Results

The mean \pm Sd age ranges of the patients and the control group were 32.0 ± 11.3 and 33.0 ± 13.2 years, respectively. There was no statistically significant difference between the ages of the patients and controls.

The average test results of neutrophils are presented in Table 1. A statistically significant increase in levels of neutrophil MDA and NO were noted in the RAS group. Also there were increased activities of CAT and SOD. No significant difference was found in neutrophil MPO, XO, and AD activities among the groups.

Table 1. Comparison of parameters of RAS and controls (Mean \pm Standard Deviation).

	RAS (n:26)	Controls (n:22)
MDA* (nmol/g prot.)	47.74 \pm 14.75	34.00 \pm 10.23
SOD* (U/mg prot.)	1.72 \pm 0.68	1.15 \pm 0.41
CAT* (k/g prot.)	18.43 \pm 6.68	12.70 \pm 4.75
NO* (nmol/g prot.)	2.64 \pm 1.25	1.69 \pm 0.76
MPO (mU/g prot.)	32.33 \pm 11.48	28.85 \pm 10.44
AD (U/g prot.)	11.29 \pm 8.55	9.56 \pm 5.25
XO (U/g prot.)	3.21 \pm 1.395	2.56 \pm 0.75

* P < 0.01

Discussion

The etiology of RAS is still unknown, but it appears to be related to an immune reaction to the oral mucosa (19). It was also demonstrated that peripheral blood neutrophils play an important role in tissue damage in RAS (10). Although the chemotactic function of neutrophils is normal in RAS, they are markedly concentrated at the ulcer area (10). In a recent study, it was (20) suggested that circulating PMNs were primed in the active RAS, as well as in the remission stage of the disease. They confirmed their hypothesis by measuring the levels of high reactive oxygen species (ROS) in the neutrophils. However, in previous studies, oxygen radical production and phagocytic function of the neutrophils have been found to be similar to that of the controls (8, 21). MPO levels are directly related to the phagocytic activity of leukocytes. When there is bacterial or viral antigen stimulus to the immune system, both the chemotactic and phagocytic activities of leukocytes increase. After phagocytosis, leukocytes produce large amounts of ROS through the action of NADPH oxidase and MPO (22). The normal levels of MPO in the neutrophils in RAS cases are correlated with the previous studies showing normal phagocytic activities of leukocytes in RAS (8, 21).

Although there are a few studies on free radical metabolism of erythrocytes and serum in RAS, to our knowledge, the oxidant/antioxidant status in neutrophils has not been studied up to now. (6, 7). In our study, neutrophilic MDA levels have been found higher than the control group. This result pointed out that there is a ROS toxicity affecting the neutrophils. Cimen *et al.* also found increased levels of MDA in serum and erythrocytes in RAS patients, but they gave explanation no about source of the oxidative stress. The source of ROS may be either activation of phagocytic cells or increased activities of ROS producing enzymes such as Xanthine oxidase and NADPH oxidase. In our opinion, the source of oxidative stress may be the NADPH oxidase of endothelial cells. Healy et al. have showed that the expression of Vascular cell adhesion molecule (VCAM-1) and E-selectin on blood vessels in RAS are likely to be important in the accumulation of inflammatory cells. (23, 24). VCAM-1 activates endothelial cell NADPH oxidase, which catalyzes production of ROS. Increase of adhesion molecules and ROS helps the extravasation of leukocytes (25). During this period, increased ROS may affect the unsaturated

fatty acids found in the membrane of the neutrophils and cause their oxidation. This process may result in an increase in the levels of lipid peroxidation end products as MDA (26).

The cellular antioxidant defense system operates through enzymatic and nonenzymatic components. CAT and SOD are the necessary enzymes for an effective defense against ROS. SOD accelerates the formation of hydrogen peroxide (H_2O_2) by using superoxide radicals. CAT is the enzyme that accelerates the degradation of unstable H_2O_2 to H_2O and O_2 . Cimen et al. found non-significantly higher activities of SOD in plasma and erythrocytes and significantly higher activity of CAT in RAS patients (7). Also Gündüz et al. found higher but non-significant erythrocyte activities of CAT and SOD (6). Increased activities of SOD and CAT in our study shows that the defense mechanisms of neutrophils were activated against increased levels of ROS which may be produced by non neutrophilic sources.

Nitric oxide is an important modulator of the inflammatory cascade. In the acute and chronic inflammatory disorders, NO has been shown to influence many pathophysiological processes, such as VCAM-1 expression, leukocyte – endothelial interaction and inflammation. Recent studies have suggested that NO may modulate cytokine induced ECAM expression (27). Since there is a leukocytic endothelial interaction and extravasation of leukocytes in RAS, increased levels of NO in neutrophils is the expected result. However, Gündüz et al. also investigated serum levels of NO in RAS patients and found no significant differences compared with the

controls. They concluded that NO did not play a primary role in the inflammatory reaction observed in RAS.

Purine nucleotides are degraded by a pathway in which AD and XO play role and uric acid is produced as an end product. XO is a well known documented biologic source of oxygen free radical and AD has been accepted as an important enzyme in maturation and function of leukocytes (28). Sogut et al. have found that AD and XO activities are significantly increased in Behçet's disease in which antigens, such as bacteria, virus and heat shock proteins induce tissue damage directly or by stimulating the leukocytes (29, 30). Normal activities of both enzymes in RAS may suggest that there is no proliferation of neutrophils. Our findings correlate with the results of Wray et al. They also found that there was no increase in the number of neutrophils in RAS (21).

In conclusion: Neutrophils may be affected by vascular adhesion molecules and endothelial ROS, and this effect can be minimized by increasing antioxidant enzymes such as SOD and CAT.

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