Oral Diseases (2007) 13, 570–574. doi:10.1111/j.1601-0825.2006.01338.x

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ORIGINAL ARTICLE

Purine catabolic enzymes and nitric oxide in patients with recurrent aphthous ulceration

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OBJECTIVES: Recurrent aphthous ulceration (RAU) is one of the most common oral mucosal disorders found in humans. Although the exact etiology of RAU is unkown, local and systemic conditions, and genetic, immunologic, and infectious factors all have been identified as potential etiopathogenic agents. The aim of our study was to compare serum xanthine oxidase (XO) and adenosine deaminase (AD) activities, and malondialdehyde (MDA), nitric oxide (NO) and uric acid (UA) levels in a group of patients affected by RAU and in a group of healthy controls.

SUBJECTS AND METHODS: A total of 26 patients with minor RAU were included in the study. Twenty-six healthy volunteers were selected to form the control group. AD and XO activities, and UA, NO, and MDA levels were studied in the serum samples of all patients and controls.

RESULTS: Serum XO and AD activities, and MDA, NO, and UA levels were significantly higher in RAU patients than in controls.

CONCLUSION: Increased XO and AD activities, NO and UA levels and lipid peroxidation were thought to take part in the pathogenesis of RAU. Hence the effects of XO inhibitors in the treatment of RAU should be evaluated in future studies.

Oral Diseases (2007) **13**, 570–574

Keywords: adenosine deaminase; malondialdehyde; nitric oxide; recurrent aphthous ulceration; uric acid; xanthine oxidase

Introduction

Recurrent aphthous ulceration (RAU), also called canker sores, is one of the most common oral mucosal disorders found in human of all ages and sex. There is no pathognomonic laboratory test, but there are clinical criteria to assist in establishing the diagnosis. There are three clinical subtypes on the basis of ulcer properties – minor, major, and herpetiform (Porter *et al*, 2000). The most common subtype is minor aphthous ulcer; recurrent, round, small, and that heal within 1–2 weeks without scarring. In addition, it can be extremely painful and may disable patients from performing their daily activities such as eating, speaking, and swallowing. The ulcers usually occur on non-keratinized oral mucosa and have erythematous halo on unattached oral mucosa (Shashy and Ridley, 2000). Epidemiologic studies indicate that the prevalence of RAU varies according to the population and geographic location. Although the exact etiopathology of RAU remains unknown, pro-inflammatory and anti-inflammatory cytokines, adhesion molecules, oxidative stress caused by free oxygen radicals, heat shock proteins, activation of leukocytes, all have been identified as likely responsible pathogenic factors (Natah *et al*, 2004).

Xanthine oxidase (XO) and adenosine deaminase (AD) are important enzymes in purine catabolic pathway. While XO catalyzes the last reaction of this pathway, it also causes production of reactive oxygen species (ROS), which causes the oxidation of macromolecules, such as lipids, proteins, and nucleic acids. AD, which catalyzes the reaction in which adenosine is deaminated to inosine, has been accepted as an important enzyme in the maturation and function of T lymphocytes (Pacheco *et al*, 2005). The main physiologic activity of the enzyme is related to lymphocytic proliferation and differentiation. As an indicator of cellular immunity, plasma activity of this enzyme has been suggested to be increased in those diseases which cause a cell-mediated immune response (Russo *et al*, 1981).

Nitric oxide (NO) is a freely diffusible inter- and intracellular messenger and synthesized from L-arginine by the family of nitric oxide synthase (NOS) enzymes in mammalian cells. Three distinct isoenzymes of NOS are known. Two of these, endothelial NOS (eNOS) and neuronal NOS isoenzymes, were named according to the tissues in which they were first identified. There is also

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Received 1 July 2006; revised 10 August 2006, 12 September 2006; accepted 14 September 2006

an inducible isoenzyme (iNOS) which generates NO in larger quantities during immunologic defense reactions (Bredt, 1999). Increased production of NO during several inflammatory processes has been recently postulated (Bosca *et al*, 2005).

The aim of our study was to compare serum XO and AD activities, and malondialdehyde (MDA), NO and uric acid (UA) levels in a group of patients affected by RAU and in a group of healthy controls.

Subjects and methods

Subjects

The study was conducted at Zonguldak Karaelmas University Medical Faculty, Departments of Dermatology, Biochemistry and Family Medicine. A total of 26 (14 male and 12 female, mean age: 34 ± 9) patients who were admitted to the dermatology outpatient clinic in a 6-month period with minor RAU which recurred not less than four times a year, and had a history of at least 1 year, were included in the study. The patients were otherwise healthy. Moreover they were not under any systemic or topical therapeutic regimen for any dermatologic or other diseases at least for the last 2 months. All patients having RAU had more than one ulcer present for not more than 48 h at the time of analysis. Twenty-six (15 male and 11 female, mean age: 33 ± 10) age- and sex-matched healthy volunteers who were admitted to the family medicine outpatient clinic for periodic health examination, found to be healthy, and also shown to be healthy by dermatology consultation formed the control group.

The patients and controls who have never used tobacco and alcohol were included in the study. The patients and the controls having any systemic disease, gingivitis or periodontal disease, or who were diagnosed to have Behçet's disease according to the diagnostic criteria of the International Behçet Committee, were excluded from the study.

Biochemical analysis

XO activity determination

Xanthine oxidase activity was assayed spectrophotometrically at 293 nm and 37°C with xanthine as substrate (Prajda and Weber, 1975). The formation of UA from xanthine results in increase in absorbency. One unit of activity was defined as 1 μmol of UA formed per minute at 37°C, pH 7.5, and expressed in U l^{-1}.

AD activity determination

Adenosine deaminase activity was estimated spectrophotometrically by the method of Giusti (1974), which is based on the direct measurements of the formation of ammonia, produced when AD acts in excess of adenosine, and expressed in U l^{-1}.

NO determination

As NO measurement is very difficult in biologic specimens, sample nitrite and nitrate levels are used as an index of NO production. The method was based on the Griess reaction (Cortas and Wakid, 1990). 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. A standard curve was established with a set of serial dilutions of sodium nitrite. Linear regression was performed by using the peak area from nitrite standard. The resulting equation was then used to calculate the unknown sample concentrations. Results were expressed as μmol l^{-1}.

MDA determination

In the samples, MDA levels were determined using the method of Draper and Hadley (1990) based on the reaction of MDA with thiobarbituric acid (TBA) at 95°C. In the TBA test reaction, MDA and TBA react to form a pink pigment with an absorption maximum at 532 nm. The reaction was performed at pH 2–3 at 95°C for 15 min. The sample was mixed with 2.5 volumes of 10% (w/v) trichloroacetic acid to precipitate the protein. The precipitate was pelleted by centrifugation and an aliquot of supernatant was reacted with 0.67% TBA in a boiling water bath for 15 min. After cooling, the absorbance was read at 532 nm. Arbitrary values obtained were compared with a series of standard solutions (1,1,3,3 tetramethoxypropane). Results were expressed as μmol l^{-1}.

UA measurement

The serum concentration of UA was measured with Roche Diagnostica kit by using the same otoanalyzer (Roche Diagnostica, Mannheim, Germany).

Statistical analysis

All values were expressed as mean ± standard deviation. SPSS for Windows 11.0 was used for statistical analysis. Student's *t*-test was used to estimate the significance between parameters. $P < 0.05$ was considered to be significant.

Results

Results are presented in Table 1. There was a remarkable increase in XO activity in patients with RAU (4.68 ± 1.46 U l^{-1}) compared with control subjects (1.47 ± 0.31 U l^{-1}) ($P < 0.0001$). AD activities in patients with RAU (20.97 ± 5.21 U l^{-1}) were found to be increased compared with controls (15.22 ± 3.93 U l^{-1}) ($P < 0.001$). In patients with RAU, significantly higher levels of serum MDA, NO and UA levels were found in comparison with the control group ($P < 0.0001$, < 0.01, and < 0.01, respectively).

Discussion

To date, measurement/evaluation of antioxidant enzyme activities (Karincaoglu *et al*, 2005) and lipid peroxidation products have been studied in samples of RAU patients associated with oxidative stress, but serum oxidant enzyme activity has never been evaluated. In this study serum XO activity was measured and higher

Table 1 XO and AD activities, and NO, MDA and UA levels of serum in RAU patients and controls

	XO ($U\ l^{-1}$)	*AD ($U\ l^{-1}$) (NV: 5–20 $U\ l^{-1}$)*	*NO ($\mu mol\ l^{-1}$)*	*MDA ($\mu mol\ l^{-1}$)*	*UA ($mg\ dl^{-1}$) (NV: $<7\ mg\ dl^{-1}$)*
Control	1.47 ± 0.31	15.22 ± 3.93	10.90 ± 1.81	0.89 ± 0.15	3.8 ± 0.6
RAU	4.68 ± 1.46*	20.97 ± 5.21*	12.54 ± 2.38**	1.12 ± 0.23*	4.7 ± 0.8**

Values are given as mean ± s.d. NV, normal values; XO, xanthine oxidase; AD, adenosine deaminase; NO, nitric oxide; MDA, malondialdehyde; UA, uric acid; RAU, recurrent aphthous ulceration.
*$P < 0.001$ compared with controls. **$P < 0.01$ compared with controls.

levels of serum XO activity were found in patients with RAU in comparison with the control group.

Xanthine oxidase is a molybdoenzyme capable of catalyzing the oxidation of hypoxanthine and xanthine in the process of purine metabolism. XO, in the presence of its substrate hypoxanthine or anthine, reduces molecular oxygen to superoxide anion radical and hydrogen peroxide, which can further react to form the more reactive hydroxyl radical, termed ROS. XO-derived ROS have been suggested to be critical factors in several mechanisms of tissue pathophysiology. XO that increases ROS production may have an important role in RAU pathogenesis with two different pathways – (i) by increasing oxidative injury/damage, or (ii) by triggering the immunologic mechanism. ROS produced by the enzyme can especially cause oxidation of membrane unsaturated fatty acids and initiate lipid peroxidation reaction chain. Oxidized lipids lose their biologic/physiologic properties and also cause oxidation of other lipids. Oxidation of the lipids initiates a process that results in impairment of structural/functional properties of the cell membrane, and lysis of the cell, and tissue damage occurs as a final result. In this study, level of MDA, which is used as a marker of lipid peroxidation, was found to be high in patients compared with the controls. This result is consistent with the current literature. Likewise, elevation of MDA was reported in previous studies in serum (Saral *et al*, 2005) and erythrocytes (Cimen *et al*, 2003) of RAU patients.

Recurrent aphthous ulceration represents a common oral mucosal disease with altered humoral and cellular immunities. Previous studies reported that inflammatory cytokine (Lewkowicz *et al*, 2005), adhesion molecule levels (Healy and Thornhill, 1999), extracellular matrix components and matrix receptors (Richards *et al*, 1996), and adhesion receptor distribution (Hayrinen-Immonen *et al*, 1992) were increased in the samples of patients with RAU. We suggest that increased XO activity is an effective factor in this pathologic process. Our suggestion is supported by a number of studies that have linked purine salvage enzymes to inflammatory responses. XO is an important mediator of inflammatory responses and cellular damage in different pathologic conditions (Borges *et al*, 2002). The enzyme has an important role in stimulating the expression of adhesion molecules by leukocytes (Sluiter *et al*, 1993) and in the activation (Kubes *et al*, 1993) and attachment of neutrophils to endothelial cells and to the extracellular matrix (Ferrel *et al*, 1992). Schwartz *et al* (1995) reported that XO-derived ROS contribute to the increased expression of mRNA for interleukin 1 beta (IL-β) and tumor necrosis factor-α (TNF-α), which are both found to be increased in RAU samples (Jurge *et al*, 2006; Sun *et al*, 2006).

Uric acid is the final product of purine metabolism in humans and it reflects XO activity. Elevated UA concentration in the RAU group compared with the controls is consistent with XO activity. UA, besides being an antioxidant molecule, constitutes a risk factor for some diseases (Ishizaka *et al*, 2006) when it reaches high levels in serum. Therefore, it has been suggested that XO inhibitor can be used in the treatment (Corry and Tuck, 2006). As both UA and XO were found to be high in our study, we think that use of XO inhibitors in the treatment of RAU can be beneficial.

In the purine metabolic pathway, AD is an important deaminating enzyme which converts adenosine and 2′-deoxyadenosine to inosine and 2′-deoxyinosine, respectively. AD is not only a cytosolic enzyme, but it can also be found as an ecto-enzyme. Serum AD activity has physiologic functions thought to be responsible for cellular immunity. AD activates T cells by binding to surface receptors. Likewise, it was reported that AD activity was found to be high in circumstances that T cell activity is increased (Sogut *et al*, 2002). In our study, serum AD activity was found to be high, supporting the opinion that cellular immunity plays an important role in the pathogenesis of RAU (Eversole, 1997). Furthermore, AD activity can be accepted as an important factor in some other alterations observed in cellular immunity, such as elevation of T cell fraction in peripheral blood (Pedersen and Ryder, 1994), elevation of Th1 activity (Dagalis *et al*, 1987) and expression (Borra *et al*, 2004) at ulcer site in RAU patients. Increased AD activity, by inducing T-cell subtypes, may play an important role in the synthesis of proinflammatory cytokines.

A strict correlation is observed between NO production and oral cavity diseases and it is suggested that elevation of NO plays an important role in the pathogenesis of these diseases (Ugar-Cankal and Ozmeric, 2006). In our study, we found significantly higher serum NO levels in RAU patients compared with the controls. There are not enough studies in the literature about the relation between RAU and NO, and there is no consensus between the few number of studies about this subject. While Gunduz *et al* (2004) reported that there is no change of NO level in RAU patients, Yildirim *et al*

(2004) reported an increase in RAU patients. A strict correlation is also observed between NO synthesis and XO activity and adenosin, a substrate of AD. Although the literature claims that increased XO activity has a deteriorating effect on NO bioavailability and NOS enzymes, Jacobi *et al* (2005) reported in their tissue culture study that, while XO decreases eNOS mRNA expression and protein amount, it upregulates iNOS mRNA. So there is an unsolved relationship between XO activity and NO production. Although it is not known whether AD activity has a direct effect on NO synthesis or not, it is claimed that there is a synergic relationship between adenosin and NO production. In this situation, it seems to be a conflict that, although as it is thought that increase in AD activity should decrease the amount of adenosin, a decrease in the amount of NO is expected, we got a contrary result. Many studies in the literature show that an increase in the amount of NO is accompanied by an increase in AD activity. A very striking example is Behcet's disease, which symptomatically resembles RAU. There are studies showing either increase or decrease of NO in Behcet's disease in the literature (Evereklioglu, 2005). In addition, IL-1β, TNF-α, γ-interferon, and IL-4 stimulate the production of NO in inflammatory cells and a combination of these cytokines has a synergistic effect on induction of iNOS (Daghigh *et al*, 2002; Ugar-Cankal and Ozmeric, 2006). As these cytokines are known to be increased in RAU (Buno *et al*, 1998), the increased serum NO level in these patients is not surprising. Besides being a physiologic modulator, NO has free radical properties. Both NO and peroxynitrite, an oxidative product of NO, can cause oxidative damage by oxidizing the cell components, such as unsaturated fatty acids. So, increased NO level, as ROS, may have some effect on the increase in serum MDA level. NO, by increasing neutrophil adhesion (Okayama *et al*, 1999), can play a role in the development of immunologic mechanisms that are thought to be responsible for the pathogenesis of RAU.

As a result, increased XO and AD activities, and NO and UA levels and lipid peroxidation were thought to take part in the pathogenesis of RAU. However, the investigation was performed at the molecular level, therefore it needs further confirmation. Hence the effects of XO inhibitors in the treatment of RAU should be evaluated in future studies.

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