Evaluation of Oxidative Stress in Erythrocytes of Guinea Pigs with Experimental Otitis Media and Effusion

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Abstract. Oxygen free radicals (OFRs) have been implicated in the pathogenesis of an increasing number of diseases and inflammatory states. They may cause cell and tissue damage by their chemical modification of proteins, carbohydrates, nucleotides, and lipids. Under physiological conditions OFRs are part of normal regulatory circuits and are neutralized by antioxidants. Infections are one cause of increased OFR production. The aims of our study were to assess whether the increased oxidative stress in experimental otitis media with effusion (OME) is reflected in erythrocytes by lipid peroxidation and to survey the alterations in oxidant and antioxidant enzyme activities in experimental OME in guinea pigs. Erythrocyte total (enzymatic plus non-enzymatic) superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), superoxide dismutase (SOD), catalase (CAT), and xanthine oxidase (XO) activities, and malondialdehyde (MDA) level were measured in 6 guinea pigs with OME and in 6 controls. The TSSA, SOD, XO activities, and MDA level in experimental OME were significantly higher than in controls. No significant differences were found in erythrocyte NSSA and CAT activities. In experimental OME induced by histamine injection, increased OFR production was observed, suggesting that OFRs may play an important role in cell and tissue damage due to OME. (received 5 December 2002; accepted 26 January 2003)

Keywords: lipid peroxidation, malondialdehyde, xanthine oxidase, otitis media, oxidative stress, histamine

Introduction

Otitis media with effusion (OME) is the most common disease of childhood with the exception of viral upper respiratory infections. OME is a leading cause of hearing loss in children [1]. The pathogenesis of OME is not fully understood.

Oxygen free radicals (OFRs), such as superoxide anion radical (O\(^{\cdot-}\)), hydrogen peroxide (H\(_2\)O\(_2\)), and hydroxyl radical (\(^{\cdot}\)OH), are highly reactive species generated by biochemical redox reactions as a part of normal cell metabolism. Oxygen metabolism in aerobic organisms has benefits, but because of the generation of OFRs, certain adverse effects also occur. Practically all the essential biomolecules can undergo oxidative reactions mediated by OFRs. The study of OFRs and their effects on biological systems has become a major area of biomedical research [2].

OFRs and lipid peroxides have been implicated in the pathogenesis of numerous diseases, eg, diabetes mellitus, cancer, rheumatoid arthritis, systemic lupus erythematosus, Behcet’s disease, infectious diseases, and atherosclerosis, as well as in aging [3-9].

Low levels of OFRs are indispensable mediators in many cell processes, including differentiation, cell cycle progression or growth arrest, apoptosis, and immunity. In contrast, high doses and/or inadequate removal of OFRs result in oxidative stress that may cause severe metabolic malfunctions and damage of biological macromolecules. Prime targets of OFRs are polyunsaturated fatty acids (PUFA) in lipid membranes. This attack causes lipid peroxidation. Increased peroxidation and decreased antioxidant protection generate epoxides that may spontaneously...
react with nucleophilic centers in the cell and thereby covalently bind to DNA, RNA, and proteins. Such reactions may lead to cytotoxicity, mutagenicity, and carcinogenicity [10].

To prevent damage caused by OFRs, multiple defense systems, collectively called antioxidants, are present in serum, erythrocytes, as well as other organs and tissues. The antioxidant system consists of antioxidant molecules (e.g., glutathione (GSH), vitamins A, E, and C, ceruloplasmin (Cp), transferrin (Trf), albumin (ALB),) and various antioxidant enzymes. Erythrocytes are excellently equipped to handle intracellular oxidative stress through the combined activity of catalase (CAT), glutathione peroxidase (GPx), and superoxide dismutase (SOD). SOD is believed to play a major role in the first line of antioxidant defense. Although CAT is not essential for some cell types under normal conditions, it plays an important role in the acquisition of tolerance to oxidative stress in the adaptive response of cells [11,12]. Xanthine oxidase (XO) functions in purine and free radical metabolism. It also catalyses the conversion of xanthine and hypoxanthine to uric acid and the production of O•-2, which is potentially toxic to cellular structures [13]. Lipid peroxidation involves oxidative conversion of polyunsaturated fatty acids to products such as malondialdehyde (MDA), which is usually measured as thiobarbituric acid reactive substances (TBARS), or lipid peroxides. Formation of MDA is one of the most studied and biologically relevant free radical reactions [14,15].

The aim of this study was to determine whether free radicals play a role in pathogenesis of OME in an animal model. We measured total (enzymatic plus non-enzymatic) superoxide scavenger activity (TSSA), non-enzymatic superoxide scavenger activity (NSSA), SOD, CAT, XO activities and MDA level in erythrocytes from guinea pigs with experimental OME and corresponding controls.

Material and Methods

Animals. This study was performed on 12 guinea pigs weighing 500-600 g. All animals received humane care in compliance with the guidelines of the Atatürk University Research Council’s criteria. The guinea pigs were fed standard laboratory chow and water. Animals were divided into 2 groups of 6 guinea pigs. Absence of middle ear disease was established by otoscopy and tympanometry. Histamine dihydrochloride (Sigma) was added to normal saline solution (1 mg/ml), and the pH of the solution was adjusted to 7.4 with sodium hydroxide. The guinea pigs were anesthetized (100 mg/kg ketamine hydrochloride and 3 mg/kg diazepam, ip); 0.1 ml of the histamine solution was injected via a 27-gauge needle through the right tympanic membrane into the middle ear cavity [16]. The same procedure was applied for control animals that received normal saline solution (0.1 ml). Three hr later, the animals were reanesthetized (100 mg/kg ketamine hydrochloride and 3 mg/kg diazepam, ip), and blood was collected by cardiac puncture after thoracotomy. The blood samples were collected in Vacutainer tubes with K3EDTA anticoagulant.

Biochemical measurements. Erythrocytes were sedimented by centrifugation and then hemolysed by 50-fold dilution with deionized water; analyses were performed on the hemolysed supernatant fraction. Hemoglobin (Hb) concentrations of the samples were measured by a GEN-S hematology analyzer. Hemolysed samples were kept at -80°C until biochemical determinations. TSSA and NSSA assays, as indicators of erythrocyte antioxidant capacity, were performed in the samples before and after adding trichloroacetic acid (TCA, 20%), as described by Durak et al [17]. First, TSSA is measured. In this method, xanthine-xanthine oxidase complex produces superoxide radicals that react with nitroblue tetrazolium (NBT) to form a farnasone compound. TSSA activity is measured at 560 nm by detecting inhibition of this reaction. By using a blank reaction in which all reagents are present except the supernatant sample and by determining the absorbance of the sample and blank, TSSA activity is calculated. Second, NSSA activity is measured in TCA-treated fractions, which are prepared by treating part of the sample with 20% (w/v) TCA solution (to removed all enzymes and proteins), and centrifuging at 5000 x g for 30 min. After the elimination of proteins by this procedure,
NSSA activity assay is performed in the supernatant fraction. SOD activity is calculated as the difference between TSSA and NSSA [17].

CAT activity was measured in hemolysates according to the Aebi method [18]. Briefly, hydrogen peroxide (H₂O₂) was used as a substrate and the decrease in H₂O₂ concentration in phosphate buffer at 20°C was followed by spectrophotometry at 240 nm. One unit of CAT activity is defined as the amount of enzyme that degrades 1 µmol H₂O₂ per min.

XO activity was determined by uric acid formation at 293 nm, as described [19]. The MDA assay, which is an important indicator of oxidant stress, was based on spectrophotometry of the pink colored product of thiobarbituric acid-reactive substances [20]. Total thiobarbituric acid-reactive substances (TBARS) were expressed as MDA, using a molar extinction coefficient for MDA of 1.56 x 10⁵ cm⁻¹M⁻¹.

Results were expressed as U/mg Hb for TSSA, NSSA, and SOD; as IU/mg Hb for CAT, and as milli-international unit (mIU)/g Hb for XO, and nmol/g Hb for MDA. One unit of TSSA, NSSA, and SOD was defined as the amount of enzyme protein causing 50% inhibition of the NBT reduction rate.

Statistics. Data were expressed as mean ± SD. Statistical significance was tested by the Mann-Whitney U-test (p <0.05 was considered significant). Statistical analyses was performed with the Statistical Package for the Social Sciences for Windows (SPSS, version 10.0, Chicago, IL).

Results

The results of this study are summarized in Table 1. As listed in the Table, the MDA level, and the TSSA, SOD, and XO activities in erythrocytes of the experimental OME group were significantly higher than those of the control group (p < 0.05, for first three parameters, p<0.01, for last one). The NSSA and CAT activities in erythrocytes of the experimental OME group did not differ significantly from those of the control group.

Discussion

Many investigators have shown that middle ear effusion (MEE) in patients with OME contains several pathogenic factors such as microorganisms, endotoxins, immune complexes, and arachidonic acid metabolites. These conditions are sufficient to induce the release of histamine that occurs in the middle ear during OME. Mast cells are generously distributed in normal middle ear mucosa. The free mast cell granules have been reported in the mucosa of OME patients, which implies ongoing degranulation in this chronic disease state. Histamine is
It has been reported that OFRs are associated with the pathogenesis of experimental otitis media [21]. OFRs are capable of reversibly or irreversibly damaging compounds of all biochemical classes, including nucleic acids, protein, free amino acids, lipids and lipoproteins, carbohydrates, and connective tissue macromolecules. These species may impair cell activities such as membrane function, metabolism, and gene expression. Propagation of damage results in a repeated chain reaction [9,22]. When the balance between OFR production and the antioxidative defense mechanisms is impaired, OFRs levels may increase. When OFRs are not removed by natural scavengers, damage occurs through peroxidation of structurally important PUFA within the phospholipid structure of the membranes [23]. Lipid peroxidation decreases both the fluidity and the barrier function of membranes, resulting in disturbances in structural organization, enzymic inhibition, and possible cell death. In addition, lipid peroxides may inhibit protein synthesis, block macrophage function, and alter chemotactic activity [24].

OFRs are known to play an important role in the intracellular killing of microorganisms by leukocytes. The challenge of polymorphonuclear cells by many activating agents, including immune complement, evokes a potent response that produces toxic oxygen species such as O•-2 and H2O2. During phagocytosis, OFRs are also produced extracellularly, but they are directly involved in inflammation. Thus, leukocytes reaching the inflammatory area produce an excessive amount of OFRs by consuming oxygen (“respiratory burst”) and OFR levels increase in the inflammation. Increased OFRs may cause cell and tissue damage [9,22,25]. Parks et al [24] reported that lipid hydroperoxide and malondialdehyde, 2 indicators of oxidative cell and tissue damage, were both significantly elevated in the middle ear mucosa of guinea pigs infected with pneumococci, in comparison with normal middle ear mucosa. Dönert et al [26] reported that MDA levels of erythrocytes and infected otitis media mucosa were significantly higher than in the control group. We found that the erythrocyte MDA level in the experimental group was significantly higher than in the control group. Our results for erythrocyte MDA level, which is an important indicator of oxidant stress, are in agreement with the results of Dönert et al [26]. These studies indicate that OFRs are increased in erythrocytes of guinea pigs with experimental OME. OFRs are known to damage the microcirculation of endothelia in all organs [24,25].

XO catalyzes the conversion of xanthine and hypoxanthine to uric acid with the production of O•-2. In this regard, XO is a key enzyme between purine and free radical metabolism. There is growing evidence that O•-2 generated by XO is primarily responsible for the cellular deterioration associated with several conditions [27]. Grisham et al [28] reported that reactive oxygen metabolites generated from xanthine oxidase and inflammatory leukocytes may play an important role in mediating mucosal injury during active episodes of ulcerative colitis. Similarly, we found that erythrocyte XO activity in guinea pigs with experimental OME was significantly higher than in the controls. The erythrocytes cannot synthesize XO de novo. The blood flow increases in inflamed regions. Possibly, XO released by injured cells may be absorbed by erythrocytes; this may account for the increased XO activity in erythrocytes of guinea pigs with OME.

SOD specifically quenches aberrant O•-2. The role of SOD in OM is not clearly understood. Sigemi et al [21] reported that plasma SOD levels in patients with otitis media were significantly elevated. We found that erythrocyte SOD and TSSA activities in guinea pigs with OME were significantly higher than in controls. Oxygen radical scavengers should be protective in inflammation. SOD is an ubiquitous and important intracellular enzyme induced by high oxygen tension; SOD is protective against oxygen radical-mediated damage. Spontaneous superoxide radical degradation usually takes place very slowly, but it occurs more rapidly in the presence of SOD, to form H2O2 [29].

It is possible that the increase we observed in erythrocyte SOD and TSSA activities could be related to the increase in O•-2 concentration produced by xanthine oxidase or from other sources. The increased SOD activity will result in an increase
in H$_2$O$_2$. The statistically insignificant change in erythrocyte CAT activity may show that this enzyme does not play an important role in OME. It is possible that the increased erythrocyte MDA level is caused by increased cellular H$_2$O$_2$ levels.

In conclusion, the high level of erythrocyte MDA is an indicator of increased lipid peroxidation, which is enhanced significantly inexperimental OME. Thus, OFRs may play an important role in the cell and tissue damage of OME.

References