CELL BIOCHEMISTRY AND FUNCTION
Cell Biochem Funct 2004; **22**: 19–22.
Published online 30 May 2003 in Wiley InterScience (www.interscience.wiley.com). **DOI**: 10.1027/cbf.1039

Lipid peroxidation, vitamins C, E and reduced glutathione levels in patients with pulmonary tuberculosis

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The present study examined the relationship between lipid peroxidation and vitamin C, vitamin E and reduced glutathione levels in plasma, erythrocytes and erythrocyte membranes of pulmonary tuberculosis patients and an equal number of age- and sex-matched healthy subjects. Enhanced plasma, erythrocytes and erythrocyte membrane lipid peroxidation with concomitant decline in vitamin C, vitamin E and reduced glutathione levels were found in pulmonary tuberculosis patients. The elevated lipid peroxidation and decreased vitamin C, vitamin E and reduced glutathione levels indicate the potential of oxidative damage to erythrocytes and erythrocyte membranes of pulmonary tuberculosis patients. Copyright © 2003 John Wiley & Sons, Ltd.

KEY WORDS — tuberculosis; lipid peroxidation; vitamin C; vitamin E; reduced glutathione

INTRODUCTION

Free radicals are continuously produced under physiological and pathological conditions. The important free radicals in biological systems are derivatives of oxygen.[1] Reactive oxygen substances (ROS) are oxidants and highly toxic to all types of biological molecules including DNA, lipids, proteins and carbohydrates.[2] The most important characteristic of toxic free radicals either *in vivo* or *in vitro* is peroxidation of lipids resulting in tissue damage and death of affected cells. Lipid peroxidation is a chain reaction providing a continuous supply of free radicals that initiate further peroxidation.[3] Free radical-induced lipid peroxidation causes marked alterations in the structural integrity and functions of cell membrane. Free radical-mediated damage has been implicated in the pathogenesis of many diseases such as cancer, atherosclerosis, inflammatory diseases and other illness.[4,5] However, normal cells and cell membranes are protected by an endogeneous defence mechanism to combat the deleterious effects of reactive oxygen substances.

By-products of lipid peroxidation formed in various biochemical reactions are normally scavenged by antioxidants. Antioxidants are compounds that are involved in effective scavenging of free radicals and in suppressing the actions of reactive oxygen substances.[5] Vitamin E, the most effective natural free radical scavenger, identified to date, is one of the most researched compounds in medicine.[6] The profound role of vitamin E in disease prevention has driven the search for reliable indices of vitamin E status. The most important extracellular antioxidant, vitamin C, has a crucial role in protection against lipid peroxidation. Vitamin C scavenges $O_2^{-\cdot}$, H_2O_2 and thiol radicals and is a potent quencher of singlet oxygen.[7] Reduced glutathione is the most powerful intracellular antioxidant and the molar ratio of reduced glutathione to oxidized glutathione serves as an important marker of the antioxidative capacity of the cell.[8]

Tuberculosis is an infectious disease caused by the bacterium *Mycobacterium tuberculosis*. Tuberculosis causes significant morbidity and mortality worldwide, with 2–3 million deaths annually and 8–10 million new cases. Although tuberculosis morbidity has decreased to low levels in developed countries, it remains one of the most common causes of morbidity

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Received 5 August 2002
Revised 18 November 2002
Accepted 9 December 2002

and mortality in developing countries including India.[9,10] In India, the incidence of tuberculosis is 1.5 million per year and about 40% of all Indians are infected with tuberculosis.[10]

The purpose of the present study is therefore to monitor the extent of lipid peroxidation and vitamins C, E and reduced glutathione status in plasma, erythrocytes and erythrocyte membranes of tuberculosis patients.

PATIENTS AND METHODS

Thirty newly diagnosed pulmonary tuberculosis patients from Rajah Muthaiah Medical College and Hospital, Annamalai University, Annamalai Nagar, India, who had not undergone any previous treatment for their tuberculosis, were chosen for the study. An equal number of healthy subjects was also investigated. The subjects were males ranging in age from 30 to 50 years. All the patients in the present study were not regular alcoholics and were clinically diagnosed as patients with tuberculosis in the right apical region of the lungs (pulmonary tuberculosis). The presence of *Mycobacterium tuberculosis* in patients was detected by carrying out a tuberculin skin test, chest X-ray, and positive sputum culture. The healthy subjects were not habituated to smoking and/or alcohol consumption and diagnosed as being free from any systemic diseases.

Fasting blood samples (7–10 ml) were obtained by antecubital vein into heparinized tubes. Plasma was separated by centrifugation at 1000 *g* for 15 min. The buffy coat was removed and packed cells were washed three times with physiological saline. The erythrocyte membrane was prepared by the method of Dodge *et al.*[11] modified by Quist.[12] The erythrocytes remaining after the removal of plasma were washed three times with 310 mM isotonic Tris-HCl buffer (pH 7.4). Hemolysis was carried out by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes which contained 20 mM hypotonic Tris-HCl buffer (pH 7.2). The erythrocyte membranes were sedimented in a high speed centrifuge at 20 000 *g* for 40 min. The supernatant was decanted and the erythrocyte membrane pellet was made up to a known volume using 0.2 M isotonic Tris-HCl buffer (pH 7.4). Aliquots from these preparations were used for the estimation of TBARS and vitamin E.

Thiobarbituric acid reactive substances (TBARS) released from endogenous lipoperoxides, reflecting the lipid peroxidation process, were assayed in plasma as described by Yagi[13] and in erythrocytes as described by Donnan.[14] The plasma was deproteinized with 10% phosphotungstic acid and treated with thiobarbituric acid at 90°C for 60 min. After cooling, 5.0 ml of n-butanol was added and the mixture was shaken vigorously and centrifuged at 1000 *g* for 15 min. The pink colour extracted in the butanol layer was read at 530 nm. The erythrocytes were deproteinized with 10% TCA and treated with thiobarbituric acid. Thismixture was heated in a boiling water bath for 15 min. It was cooled to room temperature and the pink colour which developed was measured at 535 nm.

The vitamin E level was determined by the method of Desai.[15] The lipid residue, extracted from plasma and from erythrocyte membrane, using redistilled ethanol and petroleum ether was redissolved in absolute ethanol. To this solution, ferrichloride, orthophosphoric acid and bathophenanthroline reagents were added. Vitamin E present in the lipid residue reduces ferric ions to ferrous ions and forms a pink coloured complex with bathophenanthroline–orthophosphoric acid. The absorption due to the pink complex was measured at 536 nm.

The levels of plasma vitamin C were determined by the method of Omaye *et al.*[16] The supernatant formed after the precipitation of the plasma by TCA was treated with 2,4-dinitrophenylhydrazone–thiourea–copper sulphate (DTC) reagent. The dehydro-ascorbic acid formed from the oxidation of vitamin C by copper, forms a coloured product on treatment with 2,4-dinitrophenylhydrazine whose absorbance is measured at 520 nm. Thiourea provides a mild reducing medium that helps to prevent interference from non-ascorbic acid chromogens.

The reduced glutathione level was determined by the method of Beutler and Kelly.[17] The technique involves protein precipitation by meta-phosphoric acid and spectrophotometric assay at 412 nm of the yellow derivative obtained by the reaction of the supernatant with 5-5′dithiobis-2-nitrobenzoic acid. The erythrocyte membrane protein was measured by the method of Lowry *et al.*[18]

Statistical analysis

The data are expressed as mean ± SD. Statistical comparisons were carried out using Student's *t*-test. The null hypothesis was rejected for $p < 0.05$.

RESULTS

Table 1 shows the plasma, erythrocyte and erythrocyte membrane levels of lipid peroxidation in healthy subjects and pulmonary tuberculosis patients. Lipid peroxidation as evidenced by lipid peroxide formation, was markedly increased in pulmonary tuberculosis patients as compared to healthy subjects.

Table 1. Plasma, erythrocyte and erythrocyte membrane TBARS levels in healthy subjects and pulmonary tuberculosis patients

Parameters	Healthy subjects	Tuberculosis patients
Plasma TBARS (nmole ml^{-1})	3.09 ± 0.22	8.64 ± 0.62*
Erythrocyte TBARS (pmole mg^{-1} Hb)	3.38 ± 0.28	5.90 ± 0.37*
Erythrocyte membrane TBARS (nmole mg^{-1} protein)	0.33 ± 0.02	1.56 ± 0.07*

Values are expressed as mean ± SD from 30 subjects in each group. *Significantly different from healthy subjects, $p < 0.001$.

Table 2. Plasma, erythrocyte and erythrocyte membrane non-enzymic antioxidants (vitamins C, E and reduced glutathione) levels in healthy subjects and pulmonary tuberculosis patients

Parameters	Healthy subjects	Tuberculosis patients
Plasma		
Vitamin C (mg dl^{-1})	1.38 ± 0.11	0.87 ± 0.06*
Vitamin E (mg dl^{-1})	1.11 ± 0.10	0.75 ± 0.05*
Reduced glutathione (mg dl^{-1})	48.7 ± 3.70	34.5 ± 3.20*
Erythrocyte reduced glutathione (mg dl^{-1})	52.2 ± 4.50	37.4 ± 4.01*
Erythrocyte membranes Vitamin E (μg mg^{-1} protein)	2.14 ± 0.16	1.62 ± 0.12*

Values are expressed as mean ± SD from 30 subjects in each group. *Significantly different from healthy subjects, $p < 0.001$.

Table 2 shows the levels of plasma and erythrocytes, vitamins C, E and reduced glutathione in healthy subjects and pulmonary tuberculosis patients. The levels of vitamin C, E and reduced glutathione were significantly decreased in pulmonary tuberculosis patients as compared to healthy subjects.

DISCUSSION

Tuberculosis remains one of the top three killers among infectious diseases. Tuberculosis is one of the most feared diseases in the world and about one-third of the world's population is infected with *Mycobacterium tuberculosis*.[9] In the present study, the level of TBARS was markedly increased whereas vitamins C, E and reduced glutathione levels were significantly decreased in plasma and erythrocytes of pulmonary tuberculosis patients as compared to healthy subjects.

Phagocytes undergo a respiratory burst after contact with a microorganism and huge amounts of reactive oxygen substances are produced as a consequence of this respiratory burst. Reactive oxygen substances are produced by phagocytes for the destruction of ingested microorganisms, but also contribute to inflammatory injury of the host tissue.[19] Inflammation-related oxidative stress has been implicated in the pathogenesis of lung fibrosis and dysfunction in patients with pulmonary tuberculosis.[20]

It has been well established in experimental studies as well as in patients, that the concentrations of serum lipid peroxides are increased in pulmonary inflammation.[21] Jack *et al.*[22] reported that several circulating markers of free radical activity were increased in pulmonary tuberculosis patients and some of these markers remain elevated even after completion of antimicrobial chemotherapy, indicating ongoing oxidative stress which may contribute to the development of lung functional abnormalities. Thus, we feel that the enhanced lipid peroxidation observed in the present study is due to the activation of lung macrophage by *Mycobacterium tuberculosis* infection.

Lipid peroxidation products diffuse from the site of inflammation and can be measured in the blood.[20] The lipid peroxides formed at the primary site could be transferred through the circulation to other organs and tissues and provoke damage by propagating lipid peroxidation.[23] Susceptibility of erythrocytes to peroxide stress is increased in several disease states.[24] Serum or plasma lipid peroxides play an important role in several diseases and are used increasingly as markers of tissue damage.[23] Hence the increase in the plasma lipid peroxidation observed in the present study may be due to the observed excessive lipid peroxidation in erythrocytes and the erythrocyte membranes, with consequent leakage into plasma or to excessive free radical production and diffusion from the inflammatory sites.

Antioxidants are compounds that dispose, scavenge and suppress the actions of reactive oxygen substances.[25] In the present study, the non-enzymic antioxidants were decreased in pulmonary tuberculosis patients as compared to healthy subjects. The antioxidative micronutrients vitamins C and E have been reported in several studies to regulate the production and/or reactivity of phagocyte-derived free radicals.[25] Reduced levels of serum vitamin C have been reported in patients with pulmonary tuberculosis.[26]

Vitamin E, the lipophilic antioxidant, protects cell membranes against many types of lipid peroxidation-mediated oxidative stress both by free radical scavenging and by a membrane stabilizing mechanism.[27] A positive association between vitamin E deficiency and lipid peroxide formation has been reported.[4,27] Lower levels of plasma and erythrocyte membrane vitamin E have been reported in various pathological conditions.[4,6] It has been demonstrated

that vitamin E acts as a mobilizable antioxidant, being released from tissue stores and diverted to the lungs of pulmonary tuberculosis patients during oxidative stress resulting from activation of lung macrophages and associated free radical-mediated pulmonary fibrosis.[28]

Cantin *et al.*[29] reported that glutathione has an important role in providing a component of a first line of antioxidant defence for lung parenchymal cells. Glutathione is a potent scavenger of toxic oxidants including H_2O_2, an oxidant that plays a major role in the oxidant burden placed on the epithelial cells of the lower respiratory tract in chronic inflammatory states.[29] Reduced levels of glutathione have also been reported in tuberculosis enteritis.[30]

Hence, we feel that the observed reduction in plasma and erythrocyte antioxidative nutrients (vitamins E and C) and reduced glutathione levels are mainly in response to enhanced oxidative stress in pulmonary tuberculosis patients.

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