European Review for Medical and Pharmacological Sciences 2005; 9: 361-367

Glutathione, ascorbic acid and antioxidant enzymes in the tumor tissue and blood of patients with oral squamous cell carcinoma

A.I. FIASCHI, A. COZZOLINO*, G. RUGGIERO*, G. GIORGI

Department of Pharmacology "G. Segre", Faculty of Medicine and Surgery, University of Siena – Siena (Italy)
*Department of Odontostomatologic Science, Maxillo-facial Surgery Division, Faculty of Medicine and Surgery, University of Siena – Siena (Italy)

Abstract. – *Background:* **Oral squamous cell carcinoma is one of the most common cancers in the world. Reactive oxygen species are postulated to be involved in neoplastic transformation. The antioxidant defence system limits cell injury induced by reactive oxygen species. Oxidative stress occurs when there is an imbalance between the production of reactive oxygen species and a cell's oxidant capacity or when there is a decrease in this capacity. This stress may cause mutagenesis, cytotoxicity and changes in gene expression that initiate or promote carcinogenesis.**

Objectives: **The present study was conducted to investigate whether tumor tissue and blood of patients with oral squamous cell carcinoma have altered antioxidants levels.**

Methods: **Levels of antioxidants such as reduced glutathione (GSH) and ascorbic acid (AA) and the activities of antioxidant enzymes superoxide dismutase (SOD), glutathione peroxidase (GPx), glutatione reductase (GR), were estimated in the tumor tissue and blood of 18 oral squamous cell carcinoma patients and in 20 healthy subjects as control.**

Results: **Significantly increased levels of GSH, GPx, GR and AA and significantly decreased activity of SOD were observed in tumor tissue ($p < 0.001$) and in tumor-free tissue of oral cancer patients as compared with healthy subjects. In contrast, decrease in antioxidants (GSH, GPx, GR and AA $p < 0.001$, SOD $p < 0.05$ respectively) was observed in the blood of oral cancer patients, as compared with healthy subjects.**

Conclusion: **The low levels of antioxidants in the blood of oral cancer patients may be due to their increased utilization to scavenge lipid peroxides as well as their sequestration by tumor cells. The enhanced antioxidant capacities in tumor tissues can make them less susceptible to oxidative stress, conferring a selective growth advantage on tumor cells. These findings suggest that normalization of the levels of these antioxidants might be used to reduce oral tumor malignancy.**

Key Words:

Antioxidant enzymes, Free radicals, Oxidative stress, Oral cancer.

Introduction

Cancer is a multistep disease process mediated by a diversity of endogenous and environmental stimuli. Reactive oxygen species (ROS) and other free radicals are postulated to be involved in neoplastic transformation[1].

ROS are generated endogenously and exogenously as a by-product of normal respiration and as a function of biochemical reactions using oxygen[2]. ROS at high levels are toxic to the cell, but at low levels, ROS have physiological functions, including activation and modulation of signal transduction pathways, modulation of activities of redox-sensitive transcription factors, and regulation of mitochondrial enzyme activities[3-6]. Mitochondria of normal mammalian cells are the principal endogenous sources of ROS, the main exogenous sources of ROS are drugs and other xenobiotics.

Cells have an elaborate defence system against ROS, consisting of antioxidant enzymes and low molecular weigh substances capable of scavenging many different ROS[7].

In this system, superoxide dismutase (SOD) convert superoxide radical ($O_2^{\bullet}$) into

H_2O_2, whereas glutathione peroxidase (GPx) and catalase convert H_2O_2 into water[8,9]. Therefore, two toxic species, $O_2^{\bullet-}$ and H_2O_2, are converted into the harmless product water. The removal of H_2O_2 or other hydroperoxides by GPx requires reduced glutathione (GSH) as cofactor. GPx converts H_2O_2 to H_2O and catalyzes GSH to oxidized glutathione (GSSG) simultaneously. GSSG is restored to a reduced form by glutathione reductase (GR); this reaction serves to maintaining a high GSH/GSSG ratio in the cell[9]. GR do not act on ROS directly but enables GPx to function.

Ascorbic acid (AA), a hydrophilic vitamin, is an important radical scavenger antioxidant; present in all cells can also act as a reducing agent. AA and GSH have actions in common and can spare each other under appropriate conditions[10].

Oxidative stress occurs when there is an imbalance between the production of ROS and a cell's oxidant capacity or when there is a decrease in this capacity. This stress may cause mutagenesis, cytotoxicity and changes in gene expression that initiate or promote carcinogenesis[11]. Polyunsaturated fatty acids (PUFA) are the major class of biomolecules susceptible to oxidative damage by ROS; this process is referred to as lipid peroxidation[12].

Oral squamous cell carcinoma (OSCC) is one of the most common cancers in the world. OSCC represents the 90% of the neoplasias that regard the oral mucosa and is influenced by external factors like smoke and alcohol, chronicle irritating stimuli, bad oral hygiene and nutritional deficit as fruits and vegetables[13].

Since the deleterious effects produced by ROS depend upon the balance between the oxidant and antioxidant capacity of the cells, the aim our study was to evaluate the status of the enzymatic and non-enzymatic antioxidants in patients with OSCC. Moreover, detection of antioxidants may be a useful marker in future therapeutic strategy for oral cancer.

In this paper, SOD, GPx, GR, GSH and AA were evaluated in tumor tissue, in tumor-free tissue (counter-lateral uninvolved tissue) and in venous blood of patients with OSCC and in healthy tissue and venous blood of healthy subjects.

Materials and Methods

Patients

Eighteen newly diagnosed oral cancer patients (12 females and 6 males) from Maxillo-facial Surgery Division, University of Siena, Italy who had not been previously treated for their tumors were enrolled for the study. The oral cavity tumors were histopathologically confirmed as well and moderately differentiate squamous cell carcinoma, and clinically categorized as stage III/IV OSCC. The control group comprised 20 healthy subjects (12 females and 8 males). Control tissues were taken from disease-free healthy subjects who were having impacted teeth removed, or vestibuloplasty without inflammation. Informed consent was obtained from all the participants.

Table I shows the characteristics of OSCC patients and healthy subjects.

Biological Material

Neoplastic and tumor-free tissue samples were obtained at the time of surgery and divided into 2 portions. The first of these was immediately frozen in liquid nitrogen and stored at –80° C until assayed for GPx, GR, SOD activities, levels of GSH, AA, cytosolic and total protein. The second portion was used for histologic analysis.

Table I. Characteristics of OSCC patients and healthy subjects.

Total number of healthy subjects	20
Females	12
Males	8
Age Range (years)	18-77
Total number of OSCC patients	18
Females	12
Males	6
Age Range (years)	36-87
Site	
Tongue	4
Mucosa of the cheek	3
Alveolar mucosa	6
Floor of the mouth	5
Clinical stage	
III (T3N1M0 or T3N0M0)	8
IV (T3N2M0)	10

Heparinized blood was withdrawn from the cancer patients preoperatively and from the healthy subjects. After centrifugation at 2000 × g for 10 min, the plasma was removed and erythrocytes were washed three times in 5 ml of 9 g/l NaCl solution, haemolysed by diluting fourfold with water and stored at –80° C until analyses. Hemoglobin concentration was determined by cyanmethemoglobin method[14].

Glutathione Assay

Total GSH levels in cytosol and in whole blood were analysed as described by Tietze[15]. Tissue was homogenized in EDTA K-phosphate buffer, ph 7.4 at 0° C, and 1 ml of the sample was added to an equal volume of 25% tricloroacetic acid. After centrifugation at 2000 × g for 15 minutes (0° C), the supernatant was washed with diethylether. In the remaining aliquot, proteins were assayed according to the method of Lowry[16].

Glutathione Peroxidase and Glutathione Reductase Assessment

GPx activity in cytosol and haemolysate was measured according to Paglia and Valentine using hydrogen peroxide and the rate of disappearance of NADPH was recorded spectrophotometrically (340 nm) at 37° C[17].

GR activity in cytosol and haemolysate was analysed as described by Goldberg and Spooner[18]. GR is highly specific for GSSG and NADPH remains the preferred cofactor for analytical purposes. The reaction forming GSH is strongly favored and catalytic activity is measured spectrophotometrically at 340 nm following the decrease in absorbance due to the oxidation of NADPH.

In order to measure cytosolic enzyme activity the oral tissue, samples were homogenized according to Whanger and Butler[19] in 6 volumes of cold 0.25 M sucrose in 0.1 M K-phosphate buffer pH 7.4. The homogenates were centrifuged at 40,000 × g for 20 min at 4° C and the supernatants were used for GPx and GR assays.

The cytosolic protein concentration was determined using the Lowry et al. method with BSA as standard[16].

Superoxide Dismutase Assay

Total superoxide dismutase (Cu/Zn-SOD and Mn-SOD) in cytosol and haemolysate was assayed by spectrophotometric method based on the inhibition of a superoxide-induced NADH oxidation[20]. The decrease in the rate of NADH oxidation is dependent on the enzyme concentration and subsequently saturation levels are attainable. The tissue extract was first prepared by homogenizing the oral tissue in 3 volumes of 25 nM triethanolamine-diethanolamine buffer, pH 7.4 and then cleared by centrifugation at 40,000 × g for 60 min at 4° C. The supernatant was dialysed against a cold homogenization buffer and then used for enzyme assays.

Ascorbic Acid Assay

Oral tissues were homogenized in EDTA-K-phosphate buffer pH 7.4 at 0° C. 0.5 ml of the sample (tissue or plasma) was added to an equal volume of 10% metaphosphoric acid. The samples were immediately centrifuged at 2000 × g at 0° C for 10 min. The supernatants were filtered (Anotop 0.2 mm, Merck) and 20 ml were injected in to HPLC column and analyzed as described by Ross[21].

Statistical Analysis

The significance of difference was assessed by Student's "t" test and probabilities of less than 0.05 ($p < 0.05$) were accepted as significant. The values are expressed as mean (SD).

Results

Table II shows the levels of GSH, AA and SOD, GPx, GR activities in tumor tissue, in tumor-free tissue of oral cancer patients and in oral healthy tissue of control subjects.

Activities of GPx and GR were found to be significantly increased in tumor tissue (+110,4 % and +107.1% respectively) and in tumor-free tissue (+20.6% and +56.6% respectively) when compared with healthy tissue ($p < 0.001$ for tumor tissue and $p < 0.01$ for tumor-free tissue vs. healthy tissue), whereas activity of SOD was significantly decreased in tumor tissue and in tumor-free tissue (–55.8 %, $p < 0.001$ and –12.8 % $p < 0.05$ respectively) when compared with healthy tissue.

The levels of GSH increased significantly in tumor tissue by 6-fold ($p < 0.001$) and in tumor-free tissue by 2.5-fold ($p < 0.001$) when compared with healthy tissue.

Table II. Levels of GSH, AA and SOD, GPx, GR activities in tumor tissue, in tumor-free tissue of oral cancer patients and in oral normal tissue of healthy subjects. Values are expressed as mean ± SD.

	Healthy	Tumor	Tumor Free
SOD U/ mg protein	19.37 ± 3.092	8.55 ± 1.203[A]	16.80 ± 2.772[CD]
GPx U/mg protein	12.79 ± 2.143	26.91 ± 5.001[A]	15.43 ± 3.169[BD]
GR U/mg protein	11.21 ± 1.439	23.22 ± 4.244[A]	17.56 ± 5.243[BD]
GSH nmoli/mg protein	4.39 ± 0.899	25.71 ± 4.976[A]	11.58 ± 2.219[AD]
AA nmoli/g tissue	62.59 ± 11.974	435.80 ± 80.992[A]	224.44 ± 44.196[AD]

A = as compared with healthy tissue $p < 0.001$; B = as compared with healthy tissue $p < 0.01$; C = as compared with healthy tissue $p < 0.05$; D = as compared with tumor tissue $p < 0.001$.

A similar pattern of changes in the AA levels were observed in tumor tissue and in tumor-free tissue as compared to healthy tissue.

Table III shows the circulating antioxidant status of healthy subjects and oral cancer patients. The non-enzymatic antioxidant GSH and the enzymatic antioxidants GPx, GR and SOD in the hemolysate were significantly lowered (–72.8 %, –31.1 %, –59.4 %, $p < 0.001$ and –19.4% $p < 0.05$ respectively) in oral cancer patients as compared with the control subjects.

The plasma levels of AA were also significantly lowered (–68.2 %, $p < 0.001$) in oral cancer patients as compared with the control subjects.

Discussion

Oxidative stress is due to a disturbance in the balance between the production of ROS and the efficiency of the antioxidant defense.

Table III. Levels of GSH, AA and SOD, GPx, GR activities in blood of oral cancer patients and in healthy subjects. Values are expressed as mean ± SD.

	Healthy	Cancer
SOD U/mg Hb	3.55 ± 0.422	2.86 ± 0.928[C]
GPx U/mg Hb	16.57 ± 2.526	11.41 ± 1,773[A]
GR U/Hb	18.17 ± 3.106	7.37 ± 0.964[A]
GSH µmol/ml	2.54 ± 0.331	0.69 ± 0.109[A]
AA nmol/ml	56.79 ± 9.486	18.03 ± 3.491[A]

A = as compared with blood of healthy subjects $p < 0.001$; C = as compared with blood of healthy subjects $p < 0.05$.

Thus, in oxidative stress there is an excessive production of ROS and/or there is a significant decrease or lack of antioxidant defense.

Low concentrations of ROS have been reported to stimulate cell proliferation, where as high levels can damage protein, nucleic acid, cell membrane, and to induce cytotoxicity and cell death[22,23].

In our study, we observed low levels of SOD in oral tumor tissue.

Most types of tumor cells have reduced levels of this enzyme when compared with their normal counterparts.

SOD has been proposed to be a new type of tumor suppressor gene and this concept is supported by studies demonstrating that overexpression of MnSOD in numerous transformed cell lines leads to reversion of tumorigenicity in vivo or of the malignant phenotype in vitro[24-27].

Transfection of the human MnSOD cDNA into SCC-25 oral squamous carcinoma cells significantly suppressed their malignant phenotype, leading to lower clonogenicity and growth suppression in nude mice[28].

The inhibition of proliferation and reversal of the malignant phenotype has been attributed to an increase in H_2O_2 production as a result of the dismutation reaction. Hydrogen peroxide has been shown to react with a variety of biological molecules, such as DNA and sulfhydryl group on protein, so these are potential targets. The mechanism by which hydrogen peroxide might inhibit tumor cell growth is still unknown[29].

SOD converts $O_2^{\bullet-}$ into H_2O_2, whereas GPx and catalase convert H_2O_2 into water. In this way, two toxic species, $O_2^{\bullet-}$ and H_2O_2, are converted into the harmless product water. High-

er activity of GPx resulted in decreased levels of ROS. In addition, overexpression of GPx rescues the growth suppression by MnSOD overexpression[30].

GPx plays an important role in the defence mechanisms of mammals against damage by catalyzing the reduction of H_2O_2 and a large variety of hydroperoxides into water and alcohols, respectively, con GSH as the hydrogen donor[31].

Overexpression of this enzyme was observed to protect cells against oxidative damage[32].

In the present study, we hypothesized that higher activity of GPx in oral tumoral tissue protects cells against lethal oxidative stress, causing an increase in tumor cell growth.

The removal of H_2O_2 or other hydroperoxides by GPx requires GSH as cofactor. GPx converts H_2O_2 to H_2O and catalyzes GSH to GSSG simultaneously. GSH is a tripeptide of g-glutamate, cysteine, and glycine, which is found ubiquitously in eukaryotic cells at a concentration between 1 and 10 mM. GSH has potent electron-donating capacity. Its high redox potential renders GSH both a potent antioxidant and a convenient cofactor for enzymatic reaction. This molecule is involved in the conjugation and detoxification of several types of compound that cause toxicity and carcinogenesis[9].

Because GPx requires GSH as a cofactor to exert its function, we hypothesized that higher activity of GPx can influence the levels of glutathione.

Our results show that the levels of GSH significantly increased in oral tumor tissue compared with tumor-free tissue and healthy tissue. Conversion of GSH to GSSG occurs during GPx-catalyzed reduction of H_2O_2 and other peroxides and in spontaneous reactions with free radicals[33]. GSSG is restored to a reduced form by GR; this reaction serves to maintaining a high GSH/GSSG ratio in the cell[34].

GR do not act on ROS directly but enables GPx to function.

In our study, peroxidase and reductase activities appeared to be clearly enhanced in the tumor tissue.

AA, a hydrophilic vitamin, is an important radical scavenger antioxidant; present in all cells can also act as a reducing agent.

AA and GSH have actions in common and can spare each other under appropriate conditions[10].

The increase in AA levels in tumor tissue of oral cancer patients as compared to healthy subjects may be due to further protection against ROS.

These results suggest that decrease of SOD activity and increase of AA and GSH levels as well as GPx and GR enzyme activities in oral tumor tissues may be a consequence of an increased detoxification capacity, an adaptive mechanism by which tumor cell gain a selective advantage over their surrounding normal cells[35].

Decreased SOD activity with increased GPx and GR activities in oral tumors observed in the present study is in line with similar findings in oral and in other human tumors[36-38].

Some authors considered the tumor-free tissue to be healthy, whereas others reported altered properties, possibly because of discrete changes that could have occurred from normal healthy mammary cells[39]. So, we considered that tumor cells evolved from mammary cells exhibiting comparable features as those of the tumor-free tissue.

In our study GPx and GR activities and GSH and AA levels are also increased in tumor-free tissue if compared with healthy tissue, as well as SOD activity is decreased if compared with healthy tissue. The tumor-free tissues have apparently strengthened their endogenous GPx-associated detoxification capacities. This could reflect important metabolic changes or detoxification processes against pro-oxidant event.

In contrast to tumor tissue, we found antioxidants depletion in the venous blood of OSCC patients compared to venous blood of control subjects. The erythrocytes are susceptible to ROS-induced lipid peroxidation due to continuous exposure to oxygen tension, and high content of PUFA, a target for peroxidation as well as iron, a potent catalyst for the lipid peroxidation reaction.

Decrease in antioxidant enzymes and in GSH and AA levels in blood of patients with OSCC may be due to increased scavenging of lipid peroxides as well as sequestration by tumor cells. Tumor cells have been reported to sequester essential antioxidants such as GSH to meet the demands of a growing tumor[40]. Administration of AA is known to significantly reduce the concentration the lipid peroxide and enhance GSH level in blood[40].

Moreover, the regeneration of AA requires GSH; deficiency of GSH in blood of oral cancer patients may be responsible for low level of AA in plasma of patients with OSCC.

In conclusion, our results show the following: First, an increase in the levels of GSH and GSH-system (AA, GPx and GR) in tumor tissue when compared with control tissue, which suggests that enhanced antioxidant capacities in tumor tissues make them less susceptible to oxidative stress, conferring a selective growth advantage on tumor cells. Second, an increase in the levels of GSH and GSH-system exists not only in tumor tissue but also in the tumor-free tissue. It is possible that in tumor-free tissue an increase of AA, GSH and GSH-depend enzymes reflects an enhanced detoxification capacity. Third, the decrease in antioxidant activity in blood of patients with OSCC may be caused to increased sequestration by tumor cells. Thus, if antioxidants are important in cancer, normalization of the levels of these antioxidants might be used to reduce oral tumor malignancy.

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Acknowledgements

This study was supported by a Grant from the Italy PRIN (Programmi di Ricerca Scientifica di Rilevante Interesse Nazionale).