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The oxidant/antioxidant status and cell death mode in oral squamous cell carcinoma

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Abstract

Objective. Oxidative stress and imbalance in the oxidant/antioxidant system have a critical role in carcinogenesis by affecting necrosis and apoptosis. The aim of this study is to evaluate the oxidant/antioxidant status and cell death modes in patients with oral squamous cell carcinoma (OSCC). ***Materials and methods.*** Twenty-nine patients with OSCC and 29 control subjects were included in the study. The levels of malondialdehyde (MDA), advanced oxidation protein products (AOPP) and ferric reducing antioxidant power (FRAP) were determined in plasma samples of all subjects. The necrotic and apoptotic cell death modes were evaluated with M65 ELISA and M30 ELISA, respectively. ***Results.*** MDA and AOPP values as oxidative stress markers were higher in patients with OSCC than in the control group. FRAP values evaluating plasma antioxidant status increased in OSCC patients. M65 and M30 levels indicating necrosis and apoptosis were significantly higher in OSCC patients compared to controls. There were significant correlations between MDA, AOPP and FRAP; M65 and M30 values. ***Conclusions.*** The elevated levels of oxidative stress markers together with the increase of antioxidant capacity and the presence of a strong correlation between MDA, AOPP and FRAP suggest an activation of antioxidant defense against accentuated oxidative stress determined in OSCC. Enhanced oxidation of lipids and proteins may cause decomposition of cell membranes with subsequent leakage of cytoskeletal cytokeratins as CK18 and caspase-cleaved CK18 (evaluated as M65 and M30, respectively) in the circulation, suggesting that both cell death modes are affected in OSCC.

Key Words: *Antioxidant defense, apoptosis, necrosis, oral squamous cell carcinoma, oxidative stress*

Introduction

Oral cavity cancer, one of the sub-groups of head and neck malignancies, is the sixth most frequent cancer worldwide. Oral squamous cell carcinoma (OSCC) cases constitute 86–95% of oral cavity cancers. Three quarters of all OSCC cases are from the developing world. However, the incidence has started to increase in western countries [1]. In addition, 5-year survival rates for OSCC have not shown a significant improvement in spite of recent developments in treatment methods of the disease. Thus, it keeps its characteristic as a lethal disease for over 50% of the cases diagnosed annually. The primary etiologic factors of OSCC tobacco usage and alcohol consumption are considered to be responsible for the exposure of the oral epithelium to reactive oxygen species (ROS) and free radicals [1–3]. For this reason, oral cancer can be considered as an ideal study model for evaluating oxidant/antioxidant status and the role of oxidative stress in carcinogenesis.

As an adaptive response to continuous generation of ROS in organisms, cells have an antioxidant defense system that acts to detoxify these species. The imbalance between cellular ROS production and antioxidant capability, resulting in excessive accumulation of ROS, is defined as oxidative stress. High ROS concentrations can impair cell structures including lipids, membranes, proteins and nucleic acids [4]. Malondialdehyde (MDA) occurs as a result of the lipid peroxidation. Advanced oxidation protein products (AOPP) are produced as a result of the protein oxidation in the presence of oxidative stress [5].

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The biological responses to increased and/or persisted cellular oxidative stress may manifest as DNA base alterations, strand breaks, damage to tumor suppressor genes and enhanced expression of proto-oncogenes. These responses determine whether a cell will undergo necrosis, senescence, apoptosis or will survive and proliferate. Induction of cell proliferation, decreased apoptosis and oxidative DNA damage have been proposed to be critically involved in carcinogenesis. Thus, ROS are involved in a variety of different cellular processes ranging from apoptosis and necrosis to cell proliferation and carcinogenesis [6]. Apoptosis and necrosis are cell death modes induced under different conditions. They differ in morphological and biochemical conditions [7]. Determination of apoptosis and necrosis in cancers can be performed with various methods. Cytokeratins (CK) (epithelial keratins) are attracting considerable interest in recent cancer studies as markers of cell death modes. They are mainly insoluble molecules playing an important role in cellular mechanics (cell shape, motility, division and cell-cell contact). There are two types of cytokeratins: (a) Type I (9–20) keratins that are relatively acidic and bearing a small molecular weight (40–56.5 kDa) and (b) Type II (1–8) that are relatively basic-neutral of larger molecular weight (53–67 kDa) [8]. Proliferating cells have a substantial pool of two soluble cytokeratins, namely CK8 and CK18, and their concentrations are high during the G2-M phase of the cell cycle [9]. During apoptosis, CK18 is cleaved by caspases at position relatively stable fragments [10]. These fragments can be detected in cells, sera and other body fluids being a biomarker of apoptosis, while soluble intact CK18 may be released during both necrosis and apoptosis. It is suggested that CK18 and caspase-cleaved CK18, released from necrotic or apoptotic tumor cells, can be detected by using new ELISAs M65 and M30 in the circulation of cancer patients. While M30 measures cytokeratins produced during apoptosis, M65 measures cytokeratins released from cells undergoing both apoptosis and necrosis [11].

Considering the recognized relationships between ROS, oxidant/antioxidant imbalance and the crucial role of escaping from apoptosis in the cancer etiology prompted us to evaluate the oxidative stress (detected by MDA and AOPP levels), antioxidant defense (reflected by FRAP assay) and cell death mode (apoptosis detected by M30 ELISA and both apoptosis and necrosis detected by M65 ELISA) in OSCC.

Materials and methods

Subjects

Twenty-nine patients (19 male, 10 female), mean age 58.86 ± 11.08 years with histologically proven OSCC and age- and sex-matched 29 healthy control subjects (19 male, 10 female) were included in the study. The patients had not undergone any previous treatment for their tumors which were the first malignancies diagnosed in their lives. The OSCC cases were selected from among primary tumors with different histological grades (well, moderately and poorly differentiated). The control group consisted of volunteers that had similar socioeconomic backgrounds to the patient group and had no oral lesions. The Ethics Committee at Istanbul Faculty of Medicine, Istanbul University approved the study (no: 2007/2602). Informed consent was obtained from all participants. Smoking and drinking alcohol habits of all subjects were determined. None of the subjects in the study had concomitant diseases such as diabetes mellitus, hypertension, liver disease and autoimmune disease. None of the patients or the control subjects had taken any cytotoxic medication prior to or during the study. Neither were any of them on any other medical treatment including supplementation of antioxidants.

Collection of samples

Overnight fasting venous blood samples were collected in plain tubes for routine biochemical analysis and in EDTA.K_3 for MDA, AOPP, FRAP, M65 and M30 analysis. The samples were stored at -70°C until the day on which they were analyzed for the concentrations of the study markers.

Biochemical analysis

Lipid peroxidation in the plasma was evaluated by the spectrophotometric method based on the reaction between MDA and thiobarbituric acid [12]; 0.25 ml of plasma and 0.75 ml of deionized water were combined with 2 ml reagent (26 mmol/L thiobarbituric acid, 0.92 mol/L trichloracetic acid in 0.25 mol/L HCl) and heated in boiling water for 15 min. After cooling, the flocculent precipitate was removed by centrifugation at $1000 \times g$ for 10 min. The absorbance was read at 535 nm against the blank. The MDA concentration of the samples was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$. MDA levels were expressed in μmol MDA/L plasma (nmol/mL). The intra-assay and inter-assay coefficients of variation were 4.4 and 5.7%.

AOPP levels were measured spectrophotometrically and calibrated with chloramine-T solution that, in the presence of potassium iodide, absorbs at 340 nm [5]. AOPP levels were expressed in μmol chloramine-T equivalents/L plasma. The intra-assay and inter-assay coefficients of variation were 3.9 and 5.2%.

Plasma antioxidant status was evaluated using a FRAP assay [13]. This assay uses antioxidants as reductants in a redox-linked colorimetric method. In this assay, at low pH, a ferric-tripyridylazine (Fe^{3+}-TPTZ) complex is reduced to the ferrous form, which is blue colored and can be monitored by measuring the change in absorption at 593 nm. The change in absorbance is directly proportional to the reducing

power of the electron donating antioxidants present in the plasma. The absorbance change is translated into a FRAP value by relating the change of absorbance at 593 nm of test sample to that of a standard solution with a known FRAP value. The intra-assay and inter-assay coefficients of variation were 4.2 and 5%, respectively.

The caspase-cleaved CK18 and the total CK18 were detected by commercially available ELISA kits (Peviva, Bromma, Sweden). Caspase-cleaved CK18 was measured with M30-Apoptosense assay. This assay uses a mouse monoclonal antibody detecting a neoepitope, only formed upon caspase-cleavage of CK18 at position Asp396. Therefore, values obtained by M30-Apoptosense assay represent the apoptotic cell death. Total CK18 was measured with M65 ELISA kit, which uses two mouse monoclonal antibodies specific for conventional epitopes on CK18. Thus, values obtained with M65 represent the total cell death (both apoptosis and necrosis). The detection limit of the assay for M65 was 0–2000 U/L with minimum detectable concentration 11 U/L and for M30 was 0–1000 U/L with minimum detectable concentration 25 U/L. The intra-assay and inter-assay coefficients of variation were 6.5 and 7% for M65, 7 and 9.5% for M30, respectively.

Statistical methods

Differences between groups were assessed by Mann-Whitney U-test, chi-square (χ^2) and Spearman correlation tests, where appropriate. All statistical analyses were performed with the SPSS 15.0 (SPSS Inc., Chicago, IL, USA) and NCSS 2007 statistical software package (Kaysville, UT, USA). The values were considered statistically significant if the p-value was less than 0.05.

Results

Twenty-nine patients with OSCC (19 male, 10 female) and 29 healthy controls (19 male, 10 female) were included in the study. The most common affected areas of OSCC were mandible (41.37%) and tongue (24.13%). The clinical and pathological characteristics of OSCC patients are presented in Table I. Smoking and alcohol drinking habits of controls and patients with OSCC are shown in Table II. Twenty of the patients (68.96%) used tobacco and 12 (41.37%) used alcohol. Table III shows the levels of plasma MDA, AOPP, FRAP, M65 and M30 in control subjects and patients with OSCC. There was a significant increase in plasma MDA, AOPP and FRAP values in OSCC patients when compared with healthy controls ($p < 0.0001$). Both M65 and M30 levels were also significantly higher in the patients with OSCC than in the control group ($p < 0.0001$). However, the increase of M30 in OSCC patients was more predominant in accordance with controls. Table IV presents the correlations between study parameters in patients with OSCC. There was a significant correlation between MDA/AOPP, MDA/FRAP, MDA/M65, MDA/M30, AOPP/FRAP, AOPP/M65, AOPP/M30, FRAP/M65, FRAP/M30 and M65/M30. When patients were classified according to tobacco usage, M65 and M30 values showed an increasing pattern in tobacco using ones (23% and 22%, respectively), although these differences did not reach the level of statistical significance (data not shown). When patients were classified according to alcohol usage, no significant difference in the study parameters was found. There was not any relationship between study parameters and disease grade (data not shown).

Table I. Clinical and pathological characteristics of oral squamous cell carcinoma (OSCC) patients.

	Characteristics	No. of cases
	Patients	29
	Male/female	19/10
	Age (range) (years)	58.86 (35–74)
Tumor location	Mandible (retromolar area, alveolus, floor of the mouth)	12
	Tongue	7
	Maxilla	6
	Buccal mucosa	3
	Lip	1
Histological grade of OSCCs	Well differentiated	6
	Moderately differentiated	13
	Poorly differentiated	10

Discussion

The results of the present study indicated that there was a significant increase of oxidative stress markers as MDA and AOPP in OSCC patients; plasma FRAP values, reflecting antioxidant defense, were also elevated; both cell death modes (especially apoptosis) were induced in patients with OSCC and there was a significant correlation between the study parameters.

Table II. Smoking and alcohol drinking habits of controls and patients with oral squamous cell carcinoma.

		Controls (n = 29)		Patients with OSCC (n = 29)		p
Smoking	+	6	20.69%	20	68.97%	0.0001
	–	23	79.31%	9	31.03%	
Drinking alcohol	+	5	17.24%	12	41.38%	0.041
	–	24	82.76%	17	58.62%	

Mann-Whitney U-test.

Table III. Plasma AOPP, MDA, FRAP, M65 and M30 values in healthy controls and patients with oral squamous cell carcinoma [median (range)].

	Control group	Patient group
MDA (μmol/L)	5.1 (3.6–6.1)	7.0 (5.1–10.2)*
AOPP (μmol/L)	102 (82–136)	161 (119–195)*
FRAP (μmol/L)	583 (407–733)	757 (425–985)*
M65 (U/L)	153.2 (81.4–267.0)	226.0 (113.7–1482.0)*
M30 (U/L)	76.4 (25.01–151.2)	146.0 (72.4–441.3)*

Mann-Whitney U-test; *$p < 0.0001$.
MDA, malondialdehyde; AOPP, advanced oxidation protein products; FRAP, ferric reducing antioxidant power; M65, cytokeratin CK18; M30, caspase-cleaved CK18.

The cell damage caused by oxidative stress and increased lipid peroxidation has been etiologically involved in a variety of physiological, pathological and clinical conditions including cancer [14]. Oxidative stress can arise through increased production of ROS and/or because of the deficiency of antioxidant defense. It has been revealed that patients with breast [15] or colorectal cancer [16], basal cell carcinoma of skin [17] and patients with leukemia [18] show evidence of increased lipid peroxidation. In addition, enhanced lipid peroxidation has been reported in venous blood and tumoral tissue of patients with oral squamous cell carcinoma [19,20]. Accentuated oxidative stress results in peroxidation of membrane lipids which are the most sensitive parts of the cell structure and have been considered as primary targets of oxidative stress. The increased MDA levels found in this study supported the hypothesis that cancer cells produce large amount of ROS and that oxidative stress increases in oral cancers [2,3]. The increased MDA levels could be attributed to the degradation end-products of polyunsaturated fatty acids of cell membrane lipids.

Table IV. Correlations between study parameters.

		AOPP	MDA	FRAP	M65	M30
AOPP	*r*		0.716	0.445	0.442	0.677
	p		0.0001	0.0001	0.001	0.0001
MDA	*r*	0.716		0.581	0.454	0.598
	p	0.0001		0.0001	0.0001	0.0001
FRAP	*r*	0.445	0.581		0.428	0.498
	p	0.0001	0.0001		0.001	0.0001
M65	*r*	0.442	0.454	0.428		0.705
	p	0.001	0.0001	0.001		0.0001
M30	*r*	0.677	0.598	0.498	0.705	
	p	0.0001	0.0001	0.0001	0.0001	

Spearman correlation test.

Protein oxidation follows the decomposition of the lipid structure in conditions with accentuated oxidative stress. The essential role of protein oxidation in the pathogenesis of several degenerative diseases and cancers has been recognized. AOPP are considered to be reliable markers for estimating the degree of oxidant mediated protein damage [5]. AOPP levels have been determined as increased in several studies on breast, colorectal and thyroid cancers [21,22]. Our results showing elevated plasma AOPP levels in OSCC patients were in accordance with the findings of those studies. Higher AOPP levels could be related to the decomposition (or degradation) of cellular proteins by the action of ROS on proteins in addition to changes in lipid structure. A strong correlation between MDA/AOPP, found in our study, suggests that both cellular lipids and proteins are affected by accentuated oxidative stress.

It is well recognized that there is a delicate balance between oxidative stress and antioxidant defense in healthy individuals. The antioxidant defense system counteracts the oxidative stress that induces tissue damage. There may be an inverse correlation between lipid peroxidation and antioxidant levels, although the experimental data conflicted with this view [23,24]. Significantly decreased levels of antioxidant enzymes and vitamins in cancer patients [19,22] were reported. On the contrary, elevated FRAP in some cancers [25], high plasma total antioxidant status (TAS) levels [20], as well as increased levels of antioxidant enzymes and ascorbic acid in tumoral tissue [19] of oral cancer patients have been shown. It has been seen from the results of the present study that plasma FRAP values were increased in OSCC patients. Elevated FRAP levels could be an adaptive compensatory mechanism counteracting accumulated lipid peroxides and advanced oxidation protein products. The fact that there was a significant correlation between oxidative stress markers and FRAP (FRAP/MDA and FRAP/AOPP) supported this observation.

On the other hand, increased oxidative stress can be implicated in the initiation and promotion of the carcinogenesis by causing damage to DNA—the third target of ROS after the lipid and protein structure of cells. Increased serum 8-oxoG levels (8-hydroxy-2′-guanosine—a hydroxy product of deoxyguanosine generated by the oxidative stress) in patients with oral lesions have been reported [26]. If DNA repair mechanisms (closely related to antioxidant defense) are insufficient to remove oxidative stress-related DNA damage, various mutations, activation of proto-oncogenes or suppression of tumor suppressor genes will occur, with subsequential initiation and promotion of the carcinogenesis. Increased oxidative stress and insufficiency of DNA repair mechanism determine the cellular outcome: apoptosis, necrosis or survive [27].

Some studies have revealed that as a result of the effects of oxidative stress and high ROS levels on cell cycle, apoptosis was induced [28], while others have come up with results showing that necrosis was induced [29]. Alternatively, several studies on different malignancies and on cell death modes have determined the induction of both apoptosis and necrosis in the same tissue [6]. Furthermore, it was shown that low apoptotic index is a poor prognostic factor in cancers with high malignity [30]. With regard to OSCC, some studies have revealed the presence of a disturbance in apoptosis mechanism [31,32], as well as the relationship between poor outcome and high apoptotic index [33].

There is considerable evidence that CK18 and caspase-cleaved CK18 are useful biomarkers for the determination of cell death modes of epithelial-derived tumors (carcinomas) [34,35]. It was demonstrated that CK18 and caspase-cleaved CK18, released from necrotic or apoptotic tumor cells, can be detected by new ELISAs M65 and M30 in the circulation of cancer patients [34–36]. While M30 measures cytokeratins produced during apoptosis, M65 measures cytokeratins released from cells undergoing both apoptosis and necrosis [11]. There is only one study in the literature showing increased M30 levels in patients with head and neck tumors [34]. Elevated M30 and M65 values determined in our study suggested that both cell death modes (apoptosis and necrosis) were induced in the OSCC patients. It seems likely that apoptosis in OSCC patients is more predominant in comparison with healthy controls.

As a conclusion, increased MDA and AOPP levels in OSCC suggests that oxidative stress plays an important role in the etiology of the disease. Elevation of FRAP levels probably is a compensatory response to the accentuated stress. The enhanced oxidation of lipids and proteins may cause decomposition of cell membranes with subsequent leakage of cytoskeletal cytokeratins as CK18 and caspase-cleaved CK18 in the circulation, suggested that both cell death modes (with predominance of apoptosis) were affected in OSCC.

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