Laryngeal Cancer: in Relation to Oxidative Stress

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(1), 17–23 — This study was designed to investigate the oxidative stress parameters in laryngeal cancer and cancer-free adjacent tissues. Lipid peroxidation end product and the endogenous antioxidant components-CuZn superoxide dismutase (CuZnSOD) glutathione peroxidase (GSH Px), glutathione reductase (GSSG Rd) and glutathione (GSH)-were analysed by spectrophotometric and kinetic methods. Laryngeal cancer tissue exhibited higher lipid peroxidation than cancer free adjacent tissue. CuZn SOD and GSH Px activities and GSH level were significantly higher and GSSG Rd activity significantly lower in the cancer tissue. Detection of the antioxidant status may be useful to determine the tumour resistance to therapy, to choose the correct radiotherapy/chemotherapy and to monitor the effectiveness of the therapeutic strategy. ——— lipid peroxidation; superoxide dismutase; glutathione; glutathione peroxidase/reductase; laryngeal cancer

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The human body is under constant assault by reactive oxygen molecules (ROM). These molecules with an unpaired electron in their outer orbitals are highly reactive towards and thus detrimental to cellular lipids, proteins, carbohydrates and nucleotides. As reactive oxygen molecules are capable of initiating radical chain reactions, they cause rapid spread and amplification of oxidative injury in biological systems (Gutteridge 1995).

Carcinogenic role of free radical mediated oxidative processes is well established. Oxidative stress has been reported to induce

DNA damage such as modifications of the purine and pyrimidine bases, DNA fragmentation, DNA strand breaks and apoptosis (Feig 1994; Li and Trush 1994).

Cells have an elaborate defense system against the potentially injurious oxidizing agents, consisting of antioxidant enzymes and low molecular weight nonprotein sulfhydryls. These enzymes help to keep the steady state concentrations of active oxygen at acceptable levels under physiological conditions and their inhibition can induce a prooxidant state. The vitamins C and E, β carotene and urate are

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additional physiological molecules that contribute to the natural antioxidant defense.

Induction of prooxidant state causes lipid peroxidation because the side chains of polyunsaturated fatty acids (PUFA) in membrane lipids are particularly sensitive to oxidation. Structurally unrelated classes of prooxidants and promoters are observed to cause lipid peroxidation. These findings support the notion that lipid peroxidation plays a role in carcinogenesis.

Larynx carcinoma comprises 25% all head and neck cancers and 2–3% of all malignant tumors. Epidermoid carcinoma is the most frequently encountered histologic type of larynx carcinoma, developing from the squamous epithelium that lines the larynx. Verrucous carcinoma, pseudosarcoma and basaloid carcinoma, a variant of squamous carcinoma, are rare (Adams and Maisel 1998).

Despite the presence of several reports studying the oxidative stress related parameters in cancer, they are not in good accordance with one another (El-Sharatas et al. 1993; Berger et al. 1994; Palan et al. 1994). As to laryngeal cancer specifically, there are only a few studies reported (Durak et al. 1993; Oz et al. 1994; Samir and El Kholy 1999; Yiğitbaşı et al. 2000).

We in this study aimed to observe both the extent of oxidative damage and the status of the antioxidant system directly in cancerous and the adjacent cancer-free tissues of laryngeal cancer patients. With this aim we chose to measure lipid peroxidation end product, assumed to reflect the prevailing oxidative stress and the endogenous antioxidant components-Cu Zn superoxide dismutase (CuZn SOD), glutathione peroxidase (GSH Px), glutathione reductase (GSSG Rd) and glutathione (GSH).

MATERIALS AND METHODS

Patients

Twenty patients with laryngeal cancer scheduled for surgery in Otolaryngology Depart-

ment, Cerrahpaşa Medical Faculty, Istanbul University compromised the study population of this study. The age range of the patients (18 male, 2 female) was between 39 and 70 (55.78 ± 8.94 years). Nine patients were T3, (transglottic tumors) seven were T2 (3 patients supraglottic location, 4 patients glottic origin) and four were T1 (2 patients supraglottic, 2 patients glottic location) according to the classification of the AJCC (1992) (Weymuller 1995). No lymph node metastasis was present in 15 patients. Four patients were N₁ and one was N₂. None of the patients had distant metastases. The diagnosis of cancer and staging were confirmed by computerized tomography scans, diagnostic endoscopy and histopathology. All patients had well-differentiated squamous cell cancer. Patients underwent the appropriate surgery (9 total laryngectomy, 11 partial laryngectomy) according to their conditions. Neck dissection was performed for N⁺ cases.

All the patients were smokers for about 15–25 years. Except five of them, they were moderate drinkers. Vitamin pill users were excluded from the study. A written consent for the study was obtained from each patient.

Tissue samples from the cancerous and cancer-free adjacent normal mucosa of the patients were obtained peroperatively, within 5 to 15 minutes of excision and then immediately frozen at -80°C in a light protected container until assayed. The necrotic portions of the tumor were discerned from viable portions and the center of the tumor was selected for analysis.

Preparation of tissue supernatants for the determination of lipid peroxidation end product and GSH: 10% (w/v) laryngeal tissue homogenate was prepared in 0.15 M cold KCl (pH: 7.0). The homogenate was centrifuged at 3000 rpm for 15 minutes to obtain the supernatant.

Preparation of tissue supernatants for the determination of CuZn SOD, GSH Px, and GSSG Rd activities: Tissues were homogenized

in 10 vol. of 50 mM Tris-0.1 mM EDTA (pH: 7.0) at 4°C for 30 seconds (2×15 seconds with 15 seconds cooling intervals) with a polytron homogenizer. Cytosolic fractions were prepared by centrifuging homogenates for 15 minutes at $105\,000\times g$ in Beckman ultracentrifuge.

Protein concentration was determined according to the method of Lowry et al. (1951) Assay for lipid peroxidation end product by TBA reactions (Ohkawa et al. 1979): The reaction mixture contained 0.2 ml of 10% (w/v) tissue homogenate, 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of 20% acetic acid solution adjusted to pH 3.5 with NaOH and 1.5 ml of 0.8% aqueous solution of TBA. The mixture was finally made up to 4.0 ml with distilled water and heated at 95°C for 60 minutes. After cooling with tap water, 1.0 ml of distilled water and 5 ml of the mixture of *n*-butanol and pyridine (15:1 v/v) were added and the mixture was shaken vigorously. After centrifugation at 4000 g for 10 minutes, the absorbance of the organic layer was measured at 532 nm. The level of lipid peroxidation end product was calculated using $1.56\times 10^5\text{ M}^{-1}\text{ cm}^{-1}$ as molar extinction coefficient. The results were expressed as nmol lipid peroxidation/g protein.

Materials

2-Thiobarbituric acid (TBA), sodiumhydroxid (NaOH), hydrogenperoxide (H_2O_2) were purchased from Merck Chemical Co. (Darmstadt, Germany). 5, 5'-Dithio-bis (2-nitrobenzoic acid) 3-carboxy-4-nitrophenyldisulfide: DTNB; Elman's reagent, xanthine oxidase, xanthine (2,6-Dihydroxypurine), Nitrobluetetrazolium (NBT) (2, 2'-di-p-nitrophenyl-5, 5'-diphenyl-3, 3'-(3, 3'-dimethoxy-4, 4'-diphenylene) di tetrazolium chloride, β -nicotinamide adeninedinucleotide-phosphate reduced form (NADPH) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Assay of GSH

Reduced GSH content was determined in the supernatant of the tissue homogenate according to the method of Beutler et al. (1963) using metaphosphoric acid for protein precipitation and 5,5'-dithiobis(2-nitro benzoic acid) (DTNB) as the disulfide chromogen. Results were expressed as mg/mg protein using $1.36\times 10^4\text{ M}^{-1}\text{ cm}^{-1}$ as molar extinction coefficient.

Assay of CuZn SOD activity

CuZn SOD activity was determined by the method of Sun et al. (1988). The assay involves inhibition of nitroblue tetrazolium (NBT) reduction with xanthine xanthine-oxidase used a superoxide generation. One unit of SOD is defined as the amount of protein that inhibits the rate of NBT reduction by 50%.

Assay of GSHPx activity

GSHPx activity was determined by the modified method of Paglia and Valentine (1967). Enzyme activity was determined from the oxidation of NADPH in the presence of H_2O_2 used as substrate and monitored spectrophotometrically at 340 nm. The activity of GSH Px was calculated using the molar extinction coefficient for NADPH ($\Sigma=6.22.10^3\text{ M}^{-1}\text{ cm}^{-1}$). One unit of enzyme activity was defined as 1 μmol of NADPH oxidised/min.

Assay of GSSG Rd activity

GSSG Rd activity was assayed in the supernatant of laryngeal tissue homogenate by monitoring the oxidation of NADPH at 340 nm using the method of Beutler (1975). Results were expressed as U/g protein.

Statistical analysis

All values are expressed means $\pm$ s.d. Wilcoxon signed ranks test was used for the comparison of cancer tissues with cancer-free adjacent tissues. Mann Whitney's U-test was employed for the comparison of stage grouped

cancer tissues. Correlation was determined using Pearson correlation coefficients.

RESULTS

Laryngeal cancer tissue exhibited higher lipid peroxidation end product than cancer free adjacent tissue ($p < 0.01$). With respect to antioxidant status; GSH level ($p < 0.001$) and CuZn SOD ($p < 0.001$) and GSH Px ($p < 0.001$) activities were significantly higher and GSSG Rd activity significantly lower ($p < 0.01$) in the cancer tissue.

Comparison of the stage grouped cancer tissues (stage I vs. stage II vs. stage III) revealed no significant difference. When stage I+II cancer tissues were compared with those of stage III, a significant difference was obtained in CuZn SOD activity ($p = 0.05$).

In the present study we failed to prove a correlation between the analyzed parameters and tumor staging, degree of differentiation and

lymph node metastasis.

DISCUSSION

The close relationship between free radical activity and malignancy has been extensively studied. The basic assumption appears to be that: Free radicals damage cellular materials which would result in triggering or transforming normal cells into malignant ones (Kumar 1991). Damage to DNA by oxygen-free radicals is frequently postulated to cause mutations that are associated with the initiation and progression of human cancers. Oxidants are also considered important as agents that stimulate the division rate of the tumour precursor cells (Feig et al. 1994).

Larynx carcinoma can be separated to three subgroups according to the site of the origin: supraglottic, glottic and subglottic tumors. It originates mostly from the supraglottic and glottic parts and rarely from subg-

TABLE 1. *Values of the analysed parameters (mean $\pm$ S.D.) and statistical comparison in laryngeal cancer tissue and cancer-free adjacent tissue*

	Cancerous tissue ($n=20$)	Cancer-free adjacent tissue ($n=20$)
Lipid peroxidation end product nmol/g protein	18.91 $\pm$ 9.17**	10.66 $\pm$ 5.06
GSH (mg/mg prot)	23.39 $\pm$ 5.8***	15.94 $\pm$ 5.11
GSH Px (U/mg prot)	172.92 $\pm$ 60.38***	92.82 $\pm$ 31.50
GSSG Rd (U/mg prot)	1.59 $\pm$ 0.81***	2.78 $\pm$ 1.07
Cu Zn SOD (U/mg prot)	1.54 $\pm$ 0.29**	0.65 $\pm$ 0.38

** $p < 0.01$. *** $p < 0.001$.

TABLE 2. *Values of the analysed parameters (mean $\pm$ S.D.) and statistical comparison of laryngeal cancer tissue grouped as stage I+II and stage III*

	Stage I+II ($n=11$)	Stage III ($n=9$)
Lipid peroxidation end product nmol/g protein	20.56 $\pm$ 11.24	17.07 $\pm$ 6.32
GSH (mg/mg prot)	22.63 $\pm$ 7.51	24.23 $\pm$ 3.45
GSH Px (U/mg prot)	161.72 $\pm$ 59.18	185.37 $\pm$ 62.70
GSSG Rd (U/mg prot)	1.68 $\pm$ 0.93	1.49 $\pm$ 0.70
Cu Zn SOD (U/mg prot)	1.43 $\pm$ 0.24	1.66 $\pm$ 0.30*

* $p < 0.05$.

lottic part. The supraglottic cancer is known to cause more metastasis to the neck lymphnodes than that of the glottic type. Tumors originating from different parts of the larynx behave differently. Treatment protocols vary according to the place and stage of tumor (Adams and Maisel 1998).

Lipid peroxidation, the significant characteristic feature of the radical mediated cellular damage both in vivo and in vitro, causes alteration in cell membrane fluidity, fall in membrane potential and gives rise to mutagenic lipid epoxides, hydroperoxides, alkoxyl and peroxy radicals (Samir and El Kholy 1999).

Our results of significantly high lipid peroxidation end products in laryngeal cancer tissues reflect a state of oxidative stress. Samir and El Kholy (1999) observed elevated TBARS values in the blood of laryngeal cancer patients. The increase in TBARS level especially in the postoperative period was evaluated to be partially due to surgery related tissue damage. In our previously published manuscript we observed increased erythrocyte lipid peroxides in patients with laryngeal carcinoma (Seven et al. 1999). Similar findings were observed in aerodigestive carcinoma-namely aeromaxillary, lung and oesophageal (Harada et al. 1993; Enwonwu and Meeks 1995; Takase 1995). On the other hand Yiğitbaşı et al. (2000) reported no significant difference in MDA tissue level in laryngeal squamous cell carcinoma patients.

As to our findings related to antioxidant status components; GSH level and GSHPx and CuZn SOD activities were observed to be significantly higher; whereas GSSG Rd activity lower in laryngeal cancer tissue with respect to cancer-free adjacent tissue.

Even though cancer cells generate reactive oxygen species, it has been demonstrated biochemically that antioxidant enzyme levels are low in most animal and human cancers (Oberley and Oberley 1997). Yiğitbaşı et al. (2000) reviewed 20 patients with laryngeal squamous cell carcinoma to evaluate the oxidant and antiox-

idant status on tissue level. They observed no significant difference in catalase activity; whereas a significant decrease in SOD activity. In human oral mucosa and squamous cell carcinoma it was reported that primary tumor cells displayed lower antioxidant enzyme staining intensities than did their normal cell counterparts. Moreover, poorly differentiated tumour cells showed lower antioxidant enzyme staining intensities than well differentiated tumor cells (Yang et al. 2002). On the other hand a few cancer types especially adenocarcinomas have been found to have elevated levels of antioxidant enzymes, particularly manganese superoxide dismutase and catalase (Oberley and Oberley 1997). Primary laryngeal squamous cell tumours were reported to have lower Se GSH Px activities than the matched normal mucosa. However total GSH Px activities were similar in normal and tumor tissue. (Mulder et al. 1995a). Increased activities of antioxidant enzymes were reported in leukemia (Yamanaka et al. 1979). In line with our findings, Durak et al. (1993) observed higher CuZn SOD and catalase (CAT) activities in 15 laryngeal tissues with well-differentiated squamous cell carcinoma.

Elevated levels of antioxidants may be considered as a self-serving feature of cancer cells developed to enhance their growth. CuZn SOD is reported to be the first induced enzyme. Its higher activity could be due to its induction by increased superoxide anion production. The induction of CuZn SOD in turn leads to protection of GSH Px against inactivation by O_2^- , the net effect being a higher GSH Px activity (Shull et al. 1991). In literature different results with respect to antioxidant status might rise from the facts that constitutive levels of antioxidant enzymes and their inducibility vary among tissues (Cerutti 1985; Shull et al. 1991).

It has been suggested that GSH dependent system modulates the sensitivity of the tumour to cytokines, chemotherapeutic drugs or to radiation therapy. The data of several studies on

human cancer suggest that depletion of GSH may provide an approach to sensitize tumours to chemotherapy and radiation therapy (Singh et al. 1990; El-Sharabasy et al. 1993; Perry et al. 1993).

Mulder et al. (1995b) observed mean GSH concentration in laryngeal tumours almost twice as high as in normal tissues and in nine of the ten matched pairs the tumour contained more GSH ($p < 0.05$). It is reported that normal laryngeal tissues contain almost twice the amount of glutathione S-transferase (GST) enzyme activity due to higher class alpha enzyme levels in comparison to normal oral/oropharyngeal tissues (Mulder et al. 1995b). High GSH levels and increased GST-Pi activities may contribute to inherent drug and radiation resistance in tumours. Regional metastases with very high GSH levels may even be less responsive to such therapies.

The findings of our previous study conducted in laryngeal cancer patients reveal the presence of increased blood lipooxidative damage with no change in the blood endogenous antioxidant components-GSH, GSHPx and CuZn SOD (Seven et al. 1999). Thus with regard to these facts, the assessment of the antioxidant status both in the tumour tissue and cancer-free adjacent tissue, might prove beneficial for cancer patients prior to radiotherapy/chemotherapy.

Yoshii et al. (1999) observed that human gliomas with high SOD activity tended to be resistant against radiotherapy and anticancer drugs. Thus CuZn SOD activity could be an important factor influencing the choice of oxidative cytotoxic treatment.

To conclude, in the laryngeal cancer patients, CuZn SOD and the GSH dependent system were found to be at higher activity in the cancerous tissue relative to the cancer-free adjacent tissue. Further studies are needed to investigate correlations between the antioxidant status in the tumour tissue and the resistance displayed by the tumour against the therapy. Detection of the antioxidant status may be a

useful marker not only to choose the correct therapy, but also to monitor the effectiveness of chemopreventive and therapeutic strategies and tumour recurrence.

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