EGF and TNF-α levels and oxidative/ nitrosative stress in breast and non-small cell lung cancer patients

Meme ve küçük hücreli olmayan akciğer kanser, hastalarında EGF ve TNF-α düzeyleri ve oksidatif/nitrozatif stres

Research Article

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ABSTRACT

Malignant cells exhibit increased levels of intracellular reactive oxygen species (ROS) and altered levels of antioxidant molecules. The aim of this study was to evaluate the serum levels of some antioxidants, products of lipid peroxidation and protein oxidation, growth factor and cytokine in the serum of breast and non-small cell lung cancer patients. This study includes 20 patients with breast cancer, 20 patients with non-small cell lung cancer and 20 healty subjects with no cancer as controls. Superoxide dismutase (SOD), glutathione (GSH), nitric oxide (NO), malonyldialdehyde (MDA), advanced oxidation protein product (AOPP), tumor necrosis factor-alpha (TNF-α) and epidermal growth factor (EGF) were measured in the serums. Increased levels of lipid peroxidation, protein oxidation and epidermal growth factor; low levels of NO and TNF-α were observed in cancer patients. Elevated GSH concentrations and decreased SOD activities were found in the serum of all the cancer groups. The results of our study suggest that enhanced oxidative damage altered the levels of these molecules in cancer patients.

Key Words

Oxidative stress, nitrosative stress, non-small cell lung cancer, breast cancer

ÖZET

Malign hücreler yüksek hücre içi reaktif oksijen türleri ve değişmiş antioksidan molekülleri düzeyleri sergilerler. Bu çalışmanın amacı bazı antioksidanların, lipid peroksidasyonu ve protein oksidasyonu ürünlerinin, büyüme faktörü ve sitokinin serum düzeylerini değerlendirmektir. Çalışma 20 meme kanserli, 20 küçük hücreli olmayan akciğer kanserli hasta ve 20 sağlıklı kişiden oluşmuştur. Serumlarda süperoksit dismutaz (SOD), glutatyon (GSH), nitrik oksit (NO), malondialdehit (MDA), ileri oksidasyon protein ürünü (AOPP), tümör nekroz faktör-alfa (TNF-α) ve epidermal büyüme faktörü (EGF) ölçülmüştür. Kanser hastalarında yüksek lipid peroksidasyonu, protein oksidasyonu ve epidermal büyüme faktörü düzeyleri; düşük NO ve TNF- düzeyleri gözlenmiştir. Bütün kanser gruplarında artmış serum GSH derişimi ve azalmış SOD aktiviteleri bulunmuştur. Çalışmamızın sonuçları artmış oksidatif hasarın kanser hastalarında bu moleküllerin düzeylerini değiştirdiğini öne sürmüştür.

Anahtar Kelimeler

Oksidatif stres, nitrozatif stres, küçük hücreli olmayan akciğer kanseri, meme kanseri.

Article History: Received: Sep 30, 2013; Revised: Nov 19, 2013; Accepted: Dec 9, 2013; Available Online: Dec 31, 2103.

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INTRODUCTION

Increased free radical production, or enhanced consumption of antioxidants lead to oxidative stress [1]. Despite the fact that sublethal concentrations of reactive oxygen species (ROS) are second messengers in tumor growth and metastasis [2], lethal concentrations of ROS give rise to cell death pathways in tumor cells [3]. Malonyldialdehyde (MDA) is the end product of lipid peroxidation and lead to cellular damage [4]. Advanced oxidation protein product (AOPP) is a useful marker in protein oxidative damage [5]. Nitric oxide (NO), is a product of cells in the immune system, mediates tumour vascularization and growth [6]. Human body has antioxidants such as superoxide dismutase (SOD), and reduced glutathione (GSH) which can protect against cellular and molecular damage. SOD is important in the detoxification of superoxide anion and GSH play effective role in the defence against free radicals. Disruption of the balance between the free radicals and the antioxidants may cause cellular damage and trigger carcinogenesis [7]. Neoplastic cells secrete various growth factors which have significant roles on cancer formation, generate ROS in carcinoma cells [8]. Epidermal growth factor (EGF) is involved in cancer development and proliferation. TNF-α, has a role as autocrine growth factor, causes the DNA damage and inhibits the DNA repairing in cancer. TNF-α proliferation raises in some malignant cell lines.

We evaluated markers of oxidative and nitrosative stress and antioxidants in non-small cell lung and breast cancer patients. Nevertheless we investigated effects of cancer to EGF and TNF-α levels.

MATERIALS AND METHODS

The study comprised of 3 groups: (1) control group: included 20 healthy person (2) breast cancer group (n=20) and (3) non-small cell lung cancer group (n=20). Non-small cell lung cancer patient with local advanced stage or metastatic unresectable, who had not undergone previously chemotherapy, were chosen for the study. The breast cancer patients were clinically categorised as stage II-III or metastatic after the surgical operation. These patients had chemotherapy indication but had not started the chemotherapy treatment. Patients with rheumatismal, immunologic, neurologic, cardiac, hepatic, renal, pneumonopatic except for cancer, infecte, traumatic diseases were excluded. Controls consisted of subjects with no previous history of breast cancer, non-small cell lung cancer and other cancer-releated diseases. TNF-α, EGF, NO, AOPP, GSH, MDA levels and SOD activities were examined. Human serum were collected from blood donor volunteers and then were stored at -80°C until analysis.

MDA measurement

MDA levels were determined according to the Yoshioka et al. method [9]. 250 μL serum, 1250 μL TCA trichloroacetic acid (20%), 500 μL TBA (0,67%) were mixed and heated at 95°C for 30 min. After cooling 2000 μL n-butanol were added to each samples and centrifuged at 3000 rpm for 5 min. The intensity of pink/red colour of the end product was determined at 532 nm.

AOPP measurement

Determination of AOPPs was based on spectrophotometric detection according to Witko-Sarsat et. al. [10]. Briefly, 200 μL of plasma (diluted 1:5 with phosphate-buffered saline), 200 μL of chloramin T (0-100 μmol/L) for calibration and 200 μL of PBS as blank were applied on a microtiter plate. 10 μL of 1.16 M potassium iodide and 20 μL of acetic acid were added to each well and absorbance at 340 nm was measured immediately. Concentration of AOPPs were expressed in chloramine units (nmol/L).

SOD activity assay

SOD (E.C. 1.15.1.1) activity assay was performed according to the Yi-Sun's method [11]. 2,9 mL reaction mixture (40 mL of 3mmol/ L xanthine, 20 mL of 150 μmol/L nitroblue tetrazolium (NBT) 12 mL of 400 mmol/L Na_2CO_3 and 6 mL of 1 g/L BSA), 50 μL sample and 50 μL xanthine oxidase were mixed and incubated at room temperature for 20 min After incubation 1 mL 0.8 mM $CuCl_2$ were applied and monitored spectrophotometrically at 560 nm. One unit of SOD was defined as the amount of protein which causes a 50% inhibition of the rate of NBT reduction.

Glutathione measurement

The GSH contents of samples of blood have been determined by spectrophotometrically [12]. To measurement the glutathione levels, 10% trichloroacetic acid were added into samples, mixed and allowed to stand for five minutes. After which it was centrifuged for five minutes at 3,000 r.p.m. Then 0.5 mL of the clear, protein-free supernatant, 2 mL Tris-EDTA (0.2 M, pH=8.9) and 0.1 mL 0.01 M 5,5'-ditiyo-bis-2-nitrobenzoik asit were added. After the incubated at room temperature for 10 min and monitored at 412 nm.

Nitrate and nitrite measurement

The serum was deproteinized to reduce turbidity by ethanol. Typically, a nitrate standart solution (100 μL) was serially diluted in duplicate in a 96-well. The diluting medium was used as the standart blank.after loading the plate with samples (100 μL), addition of VCl_3 (100 μL) to each well was rapidly followed by addition of the Griess reagents, SULF (50 μL) and NEDD (50 μL). Nitrite was measured in a similar manner except that samples and nitrite standarts were only exposed to Griess reagents. In either case the absorbance at 540 nm was measured using a plate reader following incubation (usually 30-45 min) [13].

Measurement of EGF and TNF-α levels

The serum TNF-α and EGF levels were detected by ELISA kits (CytElisa and Biosource respectively) according to the manufacturer's guidelines. All samples were tested in duplicate. The concentrations of TNF-α and EGF were calculated from a standart curve and expressed in pg/mL.

Statistics

All statistical analyses were carried out using SPSS statistical software (SPSS for Windows, version 17.0). All numerical data were analyzed first using the Kruskal-Wallis test to identify differences between the groups; the Mann-Whitney U-test was used to analyze two groups consecutively. Statistical significance was accepted at a value of $p<0.05$.

RESULTS AND DISCUSSION

Under normal circumstances, there is a steady balance between the production of oxygen and nitrogen derived free radicals by the antioxidant system. Any imbalance between the levels of these oxidants and antioxidants might cause DNA damage and cancer development. Many studies provided that reactive oxygen metabolites or free radicals are important in the etiology of cancer. [8, 14-16].

Damage to the breast epithelium by reactive oxygen species can lead to breast cancer. [17,18]. In literature, oxidative stress increased and anti-oxidant enzymes decreased as the disease progressed in non-small cell lung cancer patients [19]. Earlier hypothesis cited production of oxygen radicals, release of cytokines, and synthesis of prostaglandins and leukotrienes as biochemical modulators of the carcinogenic process [20].

Experimental studies have shown increased lipid peroxidation in solid tumours [21,22]. Increasing of free radical levels and decreasing of antioxidant levels were observed in malignant tissue [4]. An elevated lipid peroxide concentration was found in the tissue of all the cancer breast and non-small cell lung patients as evidenced by an increase in the mean MDA levels. The plasma MDA levels in carcinoma groups were higher when compare to control group the highest levels were seen in non-small cell lung cancer group. The increase in lipid peroxidation in cancer patients in the present study was counterbalanced by host antioxidant defence systems protecting against oxidative stress. This increase might hinge on deficiency of antioxidant molecules. Table 1 indicates the p values and biochemical parameters which was measured in this study in cancer plasma samples and controls.

Increased production of reactive oxygen species can induce SOD [23]. An increase in SOD activities due to over expression has been reported in cancer patients [24]. Recent reports suggest that oxidative stress can cause upregulation of antioxidant enzymes [25]. Several researchers reported decreases in the antioxidant level and increases in the lipid peroxidation level [26,27]. Breast cancer patients taking antioxidants showed reduced rates of recurrence, as well as less risk of mortality [28]. In the present study activity of SOD decreased in cancer patients. The results of our study suggest that free radical activity was enhanced in non-

Table 1. Levels of AOPP. EGF. GSH. MDA. NO. TNF-α. activities of SOD and values of p of the groups in the study (group 1: control. group 2: breast cancer. group 3: non-small cell lung cancer).

Parameters	Control (Group 1) (n=20)	Breast c. (Group 2) (n=20)	Lung c. (Group 3) (n=20)	p values (group1-2)	p values (group1-3)	p values (group2-3)
AOPP (nmol/ml)	85.44 ± 20.00	230.92 ± 62.41	180.35 ± 60.21	0.001	0.001	0.18
EGF (pg/ml)	16.61 ± 4.18	32.18 ± 10.24	28.86 ± 11.13	0.001	0.001	0.183
GSH (μmol/ml)	0.0063 ± 0.0021	0.028 ± 0.007	0.035 ± 0.011	0.001	0.001	0.63
MDA (nmol/ml)	3.36 ± 0.44	6.05 ± 0.60	7.30 ± 1.72	0.001	0.001	0.011
NO (μM)	54.42 ± 3.89	33.24 ± 2.52	26.47 ± 2.06	0.001	0.001	0.001
SOD (U/ml)	24.06 ± 0.99	17.44 ± 1.28	20.87 ± 1.50	0.001	0.001	0.001
TNF-α (pg/ml)	105.52 ± 17.05	60.19 ± 20.00	99.50 ± 22.89	0.001	0.231	0.001

small cell lung and breast cancer patients while the antioxidant enzyme was weakened. SOD enzyme activities were the lowest in patient with breast carcinoma. Deficient antioxidant enzyme activity gave rise to oxidative stress and celular damage.

Glutathione has regulatory effects on cell proliferation [29]. Erhola et. al. reported significantly lower total peroxyl radical trapping antioxidant potential such as protein SH-groups (thiols), levels in lung cancer patients compared to healthy controls [30]. Marika et. al. supported that lung cancer is associated with increased oxidative stress [31]. Over expression of GSH has been reported in cancer groups by us as well as by other workers [32-34]. Melloni et al. reported increased glutathione and reduced superoxide dismutase levels in non-small cell lung cancer patients compared to non-cancer controls [35]. Similarly, we found significantly higher GSH levels and lower SOD activities than the controls. The mean serum GSH levels in patients with breast or non-small cell lung cancer were higher significantly when compared to controls. We suggested that increased lipid peroxidation and host antioxidant defences associated with the development of breast and non-small cell lung cancer.

In association with cancer progress, excess lipid peroxidation and antioxidant defenses may provide the growth advantage for the tumor cells. It was thought that high levels free radicals in the cancer cells bring about over expression of antioxidant molecules and increased levels antioxidant molecules might related with sensibility of cancer cells to cancinogenic agents.

The lung cancer patients had significantly lower levels of oxidized proteins than the controls at baseline [31]. On the contrary Chandramathi et al. indicated that AOPP and MDA level was elevated in breast cancer patients compared to control subjects [36]. In the same way, in a study demonstrated that higher serum concentrations of AOPP as a marker of oxidative stress in breast cancer patients compare to controls [37]. Tu et. al., determined that significantly increased plasma levels of AOPP decreased plasma SOD activities patients with gastric cancer compared with the healthy controls [38]. Increased levels of AOPP levels were observed in cancer groups. Oxidize protein products of breast cancer group were higher when compare to non-small cell lung cancer group. AOPP serves as a useful oxidative biomarker in breast and non-small cell lung cancers.

İncreased production of nitric oxide may protect the cells from oxidative stres and this might explain the elevated levels of nitrite among lung cancer patients compared to controls [39]. Production of a potent oxidant and cytotoxic molecule, peroxynitrite in the reaction of NO with the superoxide anion may lead to increased biochemical reactivity and a wide range of damaging effects. Tamir and Tannenbaum suggest that NO has implicated in the mechanism of carcinogenesis, particularly when NO is

overproduced over a long period of time; NO may play an important role in currently accepted models of multistage carcinogenesis [20]. In a study, the pre-treatment mean levels of NO in patients were significantly higher while GSH and SOD were significantly lower as compared to control [40]. Xu et al. indicated that low concentrations of NO promoted tumour growth and proliferation [41]. In our study the NO levels were lower in breast and non-small cell lung cancer groups than the controls, were the lowest in patients with non-small cell lung carcinoma. It was not detected nitrosative stress in the cancer patients, this situation might contribute the tumor growth and proliferation. More studies are needed in this regard.

Experimental studies suggest that TNF-α lead to proliferation in some malign cell lines [42,43]. TNF-α also may role as a autocrine tumor growth factor by intracellular signaling pathway and may be effective and responsible about the formation of cancer [44,45]. It is reported that an increase in TNF-α secretion leads to apoptosis or necrosis whereas a decrease in TNF-α secretion causes the proliferation and metastasis cancer cells. Molecular details of this effects not yet known. In this study TNF-α level in cancer patients were decreased. Maybe because of this, tumor development was triggered. Reduction of TNF-α could be support escape of cancer cells from the immune system.

EGF have significant roles on cancer progression. When cancer cells are differentiated EGF uptake increases by cancer cells [46]. Significantly increased EGF levels were found in cancer groups compared to controls in this study. EGF played a role as an autocrine growth factor and stimulated tumor growth.

We investigated some biochemical parameters in breast and non-small cell lung cancer patients and thought the needed for further mechanism studies.

ACKNOWLEDGEMENT

This study was supported by Gazi University, Medical Biochemisty Laboratory. The authors would like to thank Özgür Demirbilek for the preparation of this paper.

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