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LEVELS BCL-2, P53 IN BLOOD SERUM IN SMOKERS (HEALTHY AND CANCEROUS PATIENTS).

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ABSTRACT

One of the levels protection of cells from negative effects of smoking is process apoptosis the cells contacting with cigarette smoke and change in the content of proteins regulators apoptosis. Smokers are at risk the incidence of lung cancer and respiratory tract, and according to literature data in cancer cells was found to increase the value of p53 and change Bcl-2 proteins. The purpose of study was to identify relationship between the components of the enzymatic protection and the content of apoptosis regulator proteins in smoking and in cancer associated with smoking (lung cancer and upper respiratory tract). Was studed the contens of the apoptosis markers Bcl-2 and p53 proteins in serum and the activity of the antioxidant (AO) enzymes (Cu, Zn superoxide dismutase (SOD), glutathione peroxidase (GP) and the enzyme of the biotransformation of xenobiotics - glutathione transferase (GT)) in erythrocytes. In the group of cancer patients (smokers and nonsmokers) was identified significant increasing of p53 protein levels in serum and activity of GT. Among healthy smokers were revealed two groups of donors: one with high levels OF Bcl-2, SOD and GP and another at moderate smokers with a reduced level of Bcl-2 compared with nonsmokers. These differences may be associated with the polymorphism human genes of proteins apoptosis and AO enzymes. Results suggest the relationship between the components of the enzymatic protection and proteins-regulators of apoptosis in healthy smokers and patients with cancer.

KEYWORDS: smokers, protein p53. antiapoptotic Bcl-2 protein, AO enzymes.

INTRODUCTION

Earlier was shown that smoking induces apoptosis in cells lining the respiratory tract, that caused main by the components of cigarette smoke such as nitrosamines, benzopyrene, aldehydes, aromatic amines, the other organic carcinogenic components. Combustion products involved in redox processes lead to the formation of reactive oxygen species (ROS)[1].

Due to the prolonged exposure to carcinogens and other damaging substances smoking leads to oxidative stress and gene mutations[2].

In the literature discuss the induction of the transcription group of genes associated with redox-state of cells and possible relationship between the mechanism of p53-dependent apoptosis, associated with increased production of ROS[3].

It is known that smokers are at greatest risk for lung cancer incidence and airway. Malignant transformation process is often accompanied by mutations in the p53 gene and various changes in the regulation of apoptosis, in the antioxidant (AO) enzymes and immune systems[4,5,6; 2].

Apoptosis was observed in different tissues smokers[7,8], for example, in blood leukocytes [9], where was recorded decrease Bc1-2 level and an increase in Bax and the induction of MAPKs of cascade protein kinases (ERK, INK, p38) intracellular signaling pathways. In cell lines of human lung carcinoma TW2.6 observed increased protein expression of p53 and reduction of Bcl-2, and p53 gene mutation associated with the replacement of tyrosine for cysteine[10]. In non-small cell lung cancer in tumor cells of smokers was found the elevated levels of proteins p53 and Bcl-2[6].

There are ambiguous data on the effect of smoking on the ROS generation and activity of AO enzymes : in smokers was observed as an increase in (GP)[11,12]; so and reduction (GT)[13] and activities of enzymes or lack of effect smoking on the AO enzymes[14,15].

MATERIALS AND METHODS

The experiment was performed in the Institute of biochemical physics. N. M. Emanuel Russian Academy of Sciences.

Were examined blood samples from men, consisting of 24 healthy donors (mean age 48 ± 1.5 years) and 16 cancer patients (mean age 54±2.5 years), including 10 people diagnosed with lung cancer and 6 with cancer of the larynx. Among them were the groups of smokers and non-smokers. Non-smokers and 75% of former smokers with period of smoking cessation about four years. were classified as never smokers.

Regulatory standards

Collection of venous blood samples and data processing were conducted in full compliance with the principles of World Medical Association Declaration of Helsinki "Ethical Principles for Medical Research Involving Human Subjects"(Helsinki, 1964, as amended during 1975-2000) and the current legislative and government regulations of the Russian Federation, including the Fundamentals of the Legislation of the Russian Federation on Health Protection of the Citizens,1993, and the Law of the Russian Federation "On Personal Data" N0.152-FZ, 2006. All data and the personal information collected in this study are subject to medical confidentiality and may only be brought together for processing andevaluation in an anonymous form. Blood samples were taken from all subjects following their signing the informed consent to participate in the study. The ethical approval has been obtained and informed consent was given by each subject.

Determination of p53 and Bcl-2 proteins.

The content of protein Bcl-2 was determined in the blood plasma of non-smokers (11 people) and in the blood plasma of smokers (13 people, including 4 - low level smokers (<20 cigarettes) and heavy smokers 9 persons (> 20 cigarettes per day) from healthy donors, and in cancer patients (16 people). Determination the content of p53 and Bcl-2 in plasma was performed by immunoblotting. Proteins were separated in 10% polyacrylamide gel with SDS Na by Laemmli[16]. For control of molecular weight of proteins were used SDS Protein standards (Sigma. USA). Then protein were transferred from gel on nitrocellulose membrane, membrane was cut into two strips containing proteins with a molecular weight of 53 kDa (p53) and 25 kDa (Bcl-2), respectively and determination content of proteins was carried out separately.

Antibody to p53 and Bcl-2.

The first antibody on p53 was FL-393 (Santa Cruz. USA). The second antibody was peroxidase labeled sc-6243 (Sigma).

As the first antibody for determination of Bcl-2 protein were used Monoclonal Anti-Bcl-2 clone 10C4, as second antibody - anti-rabbit IgG labeled horseradish peroxidase (Sigma). To determine position of proteins on a nitrocellulose membrane were used a Staining Kit of AES (Sigma Aldrich). Optical density of bands corresponding proteins was measured by the BMPscale program.

Determination of SOD, GP, GT.

Activity of AO enzymes were determined in erythrocytes. SOD activity was determined by inhibition in the reduction reaction nitro blue tetrazolium (Serva. Germany) superoxide radicals generated by xanthine oxidase in the oxidation of xanthine. For the unit of activity took the amount of enzyme resulting in 50% inhibition of the rate of formation of formazan.[17]

Activity was related to 1 mg of hemoglobin (Hb). The activity of GP was determined by the oxidation of the reduced nicotinamide adenine dinucleotide phosphate (NADP) glutation reductase in a coupled reaction using as a substrate of hydroperoxide tert-butyl[18]. The activity of GR by oxidation of the reduced NADP in the presence of oxidized glutathione[19].

Catalase activity was determined by decomposition rate of the hydroperoxide hydrogen[20]. 1 unit of activity of GP, GT and catalase took the amount of enzyme catalyzing the conversion of 1 MK mole of substrate in 1 min. activity of all enzymes (except SOD) carried by 1 g Hb. Hb content was determined by the method of Van Kampen[21].

Statistics

The results were processed using Statistica. version 6. The group was characterized by average values of measured values and the corresponding standard deviations Statistically significant differences between parameters was evaluated by the Mann-Whitney test. Correlations between variables were evaluated by the method of Spearman. The level of significance was taken as $p < 0.05$.

The following reagents were used: potassium carbonate, methanol, (Merck, Germany), sucrose, Tris, EDTA (Ethylenediaminetetraaceticacid), FCCP (carbonyl cyanide-*p*-trifluoromethoxyphenyl-hydrazone), malate, glutamate, succinate (Sigma, Aldrich, USA), Hepes (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Biochemica Ultra, for molecular biology) (MB Biomedicals, Germany).

RESULTS AND DISCUSSION

As known, smokers have a high incidence of lung cancer and the respiratory tract, and among a number of genetic abnormalities that are involved in the pathogenesis of human cancers, the most common are the P53 gene mutations. According to some sources in the lung cancer cells was found increased apoptosis markers proteins p53 and Bcl-2 on 51% and 27% respectively.

We investigated content of protein regulator p53 in plasma for two groups of donor, healthy patients (8 people) and cancer patients (7) (Fig. 1.b). It is noted that they demonstrated high variability of content p53 in the control group (from 0.8 to 3.2, for one individual the content of p53 was equal to 5.7), but these variations did not correlate with intensity of smoking.

We also compared the level of p53 in groups of healthy donors and cancer patients without consideration of smoking habits. As can be seen from Fig. 1,b, the content of protein p53 in cancer patients was significantly higher than in healthy group and more than 3 times exceeded the level of p53 in the group of healthy donors (p = 0.0002).

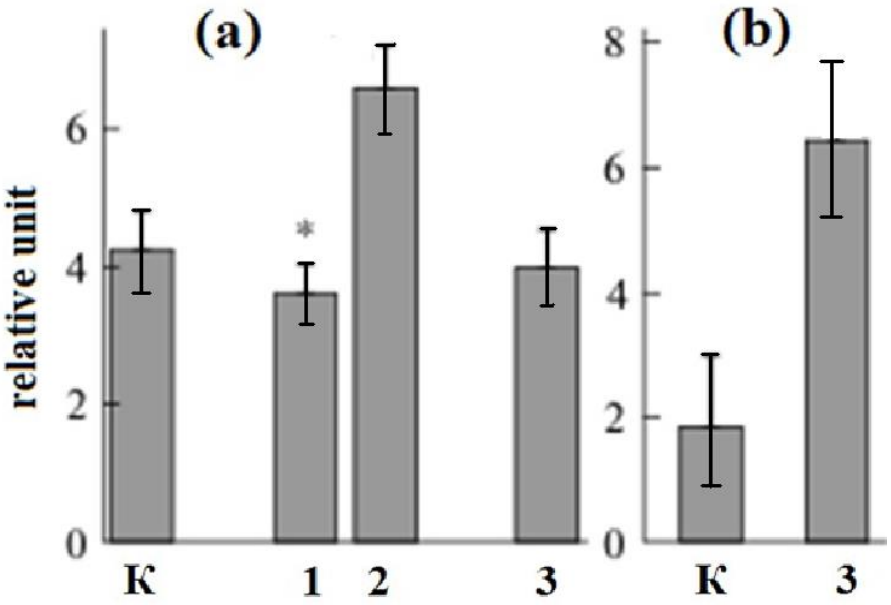

Figure.1: The content of the protein Bcl-2 (a) and p53 (b) in different studied groups of donors and cancer patient. a : К- non-smoking donors ,1 – smoking donors 1-type, 2 - smoking donors 2- type, 3 - cancer patients. b: К- donors, 3- cancer patients. Significant differences from non-smoking donors, p<0.05.

We also detected that among cancer patients the level of p53 in plasma varied slightly, and content of protein p53 in the blood plasma smokers and nonsmokers patient did not differ significantly.

'Enhance of content of protein p53 in cancer patients is consistent with the literature data and was observed in the row of cancer diseases, such as hepatoma of the liver associated with chronic infection with hepatitis C virus[22] and tumors of the upper respiratory tract and lungs[23,24]. Excessive accumulation of p53 protein may be associated with a point mutation in the P53 gene, leading to the appearance of p53[25] a long-lived protein. Also was shown an increasing content p53 in serum wumen with breast cancer stage III and IV[26]. Currently determination of serum protein p53 has become now one of the methodes used in the clinic to determine the presence of malignant tumors[27].

We also studed the content of antiapoptotic protein Bcl-2 in plasma of healthy donor and in cancer patients. Was foud that in groups of cancer patients and healthy donors, the differences between the values of the Bcl-2 for non-smoking (6 people) and smokers (10 people) of cancer patients were not statistically significant (p >0.05). The content of Bcl-2 in the blood plasma of cancer patients was (4.05 ± 0.5) (Fig. 1a, 4).

Was found (Fig. 2.b) that in the group of cancer patients activity of GT were increased compared to the activity of GT in healthy donors (p = 0.032). The literature provides similar data about the increase in the activity of GT in oncology, for example, in red blood cells of patients with cancer of the oral cavity.[28] It is known that GT is included in the detoxification of carcinogens including in It was established that a special role in the response to oxidative stress, inflammation, apoptosis plays a signaling pathway Nrf2 - Keapl, which regulates the activity and gene expression of AO enzymes (including SOD and GP) and enzymes of detoxication of xenobiotics (GT), the promoters of which contain the antioxidant-responsive elements, adjustable by varying the redox-sensitive transcription factors such as p53, NF(kappa)B, AP-I, and Nrf2[28-30] tobacco smoke.

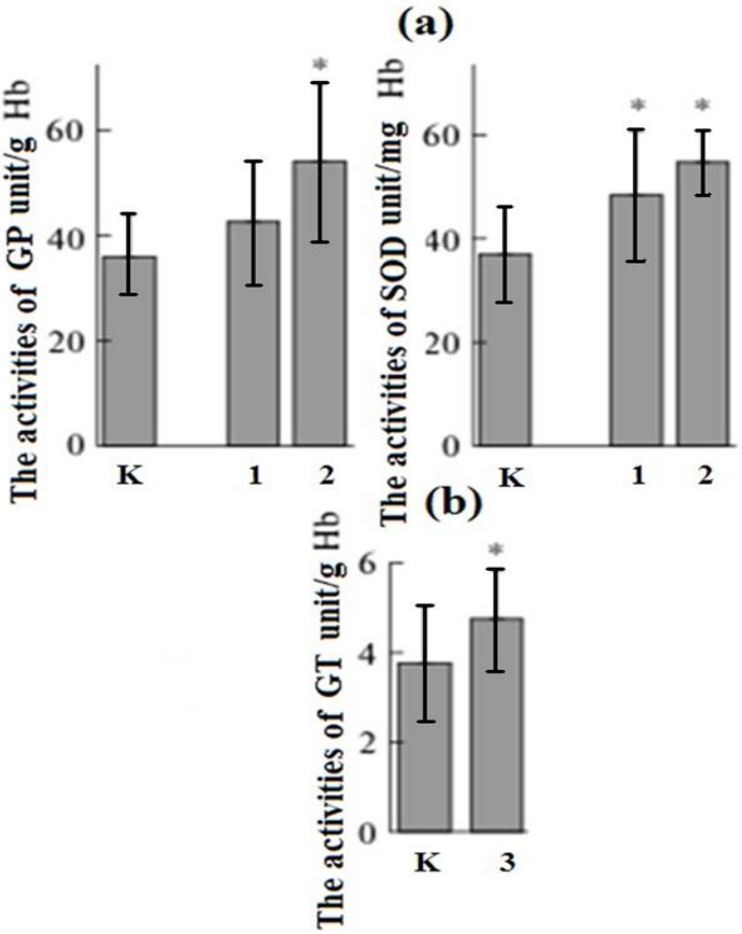

Figure 2: The activities of SOD and GP(a), GT(b) in the different groups examined donors and cancer patients: a) K-non-smoking donors, 1-smokers donor type 1, 2-mokers donor type 2, 3- cancer patients,* - significant differences from non-smoking donors, p <0.05; b) K –Donors, 3- cancer patients,* - significant differences from donors, p <0.05.

The role of GT in the modulation of risk associated with smoking to lung cancer and cancer of the upper respiratory tract[28] is currently the subject of intensive research.

Thus, the group of examined cancer patients is characterized by elevated protein levels of p53 and the activity of GT. These indicators can be of prognostic importance in choosing treatment strategies for patients with cancer of the head and neck[31]. It has been shown that increased expression of both markers is a negative indicator in the case of radio-chemotherapy.

The amount of the antiapoptotic protein Bcl-2 in plasma was determined in non-smokers and smokers of healthy donor (Fig. 1a).

Large differences were found in smokers donors, which allowed to divide this group on two subgroups: (subgroup of type 1) having a low content of Bcl-2 (3.7= 0.39) (Figure A 1 a. 2), and (subgroup type 2) containing only a few (5 people) heavy smokers donors with a high content of Bcl-2 (0.57).

The lowering content Bcl-2 in smokers of 1 type compared in non-smokers donors ($p = 0.027$) could indicate a strengthening of the process of apoptosis in smokers. This is consistent with the work[9], where demonstrated a high level of apoptosis in leukocytes of blood cells in smokers, which was accompanied by a decrease of antiapoptotic protein Bcl-2 and an rise proapoptotic Bax level.

At the same time we detected an increase activity of SOD and GP in smokers the 1st and 2nd type, compared with a group of non-smokers. In the group with the highest value of Bcl-2 (Fig. 2a) there is a significant increase (1.5 times) the activities of SOD ($p = 0.007$) and the GP {$p = 0.015$) when compared with non-smokers (Fig. 2a). In the group with a low Bcl-2 (Fig. 2a) also found a increase in SOD activity ($p = 0.05$) and a trend towards increased activity GP {$p = 0.17$).

Thus, we shown that for some heavy smokers increase of the antiapoptotic protein Bcl-2, correlates with an increase of the activity SOD and GP included in the process of detoxification of ROS.

Higher level of Bcl-2 of heavy smokers (type 2) can be explained by the presence in some people of genetically determined adaptive mechanism, which leads to increased protective reaction on long-term high level of apoptosis of heavy smokers. This is consistent with the anti-inflammatory[33] and AO function of family Bcl-2 protein in the blood[34,35]. So, in Amstad work[35] was shown that in cells overexpressing Bcl-2 the level of hydroxyl radicals decreased, glutathione levels increased, lipid peroxidation inhibited.

Apparently, multidirectional responses to adverse effects in groups of smokers 1 and type-2 may be associated with the polymorphism human genes of proteins apoptosis and antioxidant defense, which regulate cell response to the oxidative stress.

SUMMERY, CONCLUSION AND RECOMMENDATION

Thus the definition of p53 protein levels in plasma and the activity of GT in erythrocyte may be useful in clinic for detection of the malignization process and monitor the treatment. So, earlier was detected that high level p53 in serum of breast cancer patients (women) decreased after the doxorubicin treatment (36 up to 54 years), and was the negative impact of the treatment more older women[26].

Among healthy heavy smokers was identified two group of donors with different levels of protein Bcl-2, activities of SOD and glutathione peroxidase (GP) .We can assume that in smokers with high level of Bcl-2 the processes reparation of the cell will occur more intensively The high level of Bcl-2 of some heavy smokers can help reduce the formation and accumulation of ROS and interfere to malignancy of cells.

The relationship between content of Bcl-2 protein in serum and of processes reparation DNA was noted in the works[36,37]. So, injection antioxidant phenozan K (10-14M ,10-4M) to mice caused induction Bcl-2. and p53 in the serum[36], and led also to a decrease in the content of double-strand DNA breaks in cells[37] At the same time phenozan K in ultralow doses was highly effective in the treatment tissue hypoxia of newborn, and resulted to an early normalization of the phospholipid composition and activity of antioxidant defense enzymes in brain.[38]

The results show the relationship between content of proteins regulators of apoptosis in blood and AO enzymes in erythrocyte in healthy smokers and in patients with cancer that may be used as prognostic factor and can help to monitor the process of treatment.

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