

THE EVALUATION OF PROOXIDANT AND ANTIOXIDANT STATE OF TWO VARIANTS OF LEWIS LUNG CARCINOMA: A COMPARATIVE STUDY

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Aim: To study the functional activity of enzymatic component of antioxidant system and to evaluate an intensity of prooxidative processes in Lewis lung carcinoma variants (LLC and LLC/R9). **Methods:** Activity of glutathione-S-transferase (GST), glutathione peroxidase (GP), glutathione reductase (GR), catalase (Cat), content of lipid peroxidation (LP) byproducts were analyzed in tumor extracts at the 24th day after tumor transplantation, extracts of muscle tissues of tumor-bearing mice and intact mice using the method of optic spectrometry. **Results:** It was revealed higher level of GST and GR activities, and lower level of GP activity, and a tendency for decrease of Cat activity level in LLC/R9 tumors compared to LLC tumors. The content of primary LP products was higher in LLC/R9 tumors while two tumor variants didn't differ significantly by the content of tiobarbiturate-active products. **Conclusion:** The pool of reduced glutathione in LLC/R9 tumors is more effectively replenished with GP involvement and is used by GST for detoxification of exogenous xenobiotics compared with LLC. Lower GP activity and the tendency to decreased Cat activity is characteristic property for LLC/R9 tumors compared to LLC.

Key Words: Lewis lung carcinoma, antioxidant enzymes, lipid peroxidation, metabolic stress, resistance to cisplatin, glutathione-dependent enzymes.

In contrast to untransformed cells the microenvironment of malignant cells in tumor is characterized by the deficiency of nutrient substrates (oxygen, glucose, amino acids etc.) and by accumulation of waste products of tumor cells. Arising in the conditions of oxygen deficiency in tumor due to the insufficient level of vascularity the metabolic stress is followed by the accumulation of reactive oxygen species (ROS) that display the damaging activity towards macromolecules and biological membranes, from the one hand, and stand as the regulatory components of signal pathways that regulate proliferation, differentiation, apoptosis, autophagy and neoangiogenesis etc., from the other hand [1–3, 6, 8, 10, 21]. Thereby on the background of metabolic stress the survival of tumor cells substantially depends on the functioning of antioxidant system involved in the regulation of prooxidant processes intensity. The maintenance of the prooxidant–antioxidant balance and the regulation of ROS content is realized in the cell in response to metabolic stress by the functioning of multi-level systems of enzymatic and non-enzymatic antioxidant defenses [9]. Glutathione-dependent system belongs to the key components of antioxidant defense system that take part not only in neutralization of lipoperoxides but also play an important role in the regulation of functional activity of

mitochondrial antiapoptotic proteins (Bcl-2 family) and in irreversible inactivation of protein kinase C [27, 29]. The key constituent of glutathione system is glutathione (γ -glutamyl-L-cysteinyl-glycine), the intracellular pool of which includes the reduced (GSH) and oxidized (GSSG) forms, compound bisulfides (GS-S) and thioesters [7, 23]. GSH/GSSG ratio maintained due to the activity of these enzymes is accepted to be considered as an important index of intracellular redox-state. The avoidance of redundant ROS accumulation in the cells occurs owing to the functioning of selenium-dependent (for H_2O_2) and selenium-independent (for organic peroxides) glutathione peroxidases [17]. Glutathione reductase provides the reduction of GSSG using NADPH and regulates the amount of substrate accessible for glutathione-S-transferase and glutathione peroxidase [15]. Catalase (E. C. 1.11.1.6) possesses the similar to glutathione peroxidase functional activity and is localized in peroxysomes in the form of tetrameric haemoprotein decomposing hydrogen peroxide that is known to cause the direct induction of Atg proteins expression and to stimulate the autophagic cell death [11, 13, 30]. The regulation of this enzyme's activity by the modifications of the active or regulatory sites and by the selective degradation of catalase is pivotal in autophagy induction as one of the adaptive mechanisms in response to the nutrients deficiency at the high requirements in the energy supply of vital functions [30]. In this connection, the study of functional activity of antioxidant system is important for the establishment of the possible mechanisms of adaptation and survival of tumor cells in the conditions of permanent hypoxia and nutrients deficiency.

The aim of our study was to evaluate the connection between the indices of functional activity of glutathione-dependent enzymes in tumors of two Lewis lung carcinoma variants and the sensitivity of these tumors to metabolic stress.

Received: April 26, 2010.

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Abbreviations used: Cat – catalase; CMT – control muscle tissue; EFS – embryonal fetal serum; GDNB – glutathione-S-2,4-dinitrobenzene; GP – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; GST – glutathione-S-transferase; IMT – intact muscle tissue; LLC – Lewis lung carcinoma; LLC/R9 – modified variant of Lewis lung carcinoma; LP – lipid peroxidation; ROS – reactive oxygen species; TBAP – tiobarbiturate-active products.

MATERIALS AND METHODS

The experiments were performed using 20 males of mice in the age of 2–2.5 months with the weight of 18–23 g. All experimental procedures were conducted following the normative rules of bioethics. In the study the tumor cells of two variants of Lewis lung carcinoma were used. The variants differ in the sensitivity to the deficiency of nutritive substrates: (i) the cells of the wild-type of Lewis lung carcinoma (LLC) are resistant to the metabolic stress; (ii) the cells of modified variant of carcinoma (LLC/R9) display the higher sensitivity to low level of nutrients (in comparison with LLC) [18, 24]. Tumor cells were cultivated in RPMI-1640 (“Sigma”, USA) with addition of 10% EFS (“Sigma”, USA), 2 mM L-glutamine and 40 µg/ml gentamycin at +37 °C in humid conditions with 5% CO₂. Then cells were inoculated to mice in the muscle of dextra thigh in the amount of 1·10⁶ cells in 0.1 ml of Haenks’ solution per animal.

On the 24th day after tumor transplantation the euthanasia of animals under the light ester anesthetic was performed. Tumors and thigh muscles (CMT — control muscle tissue) were removed. Since tumor cells were transplanted into thigh muscle of the analogous tissue without tumor was considered as the control tissue. As the control tissue the muscle tissue from the thigh of intact mice was used (IMT — intact muscle tissue). Tumors and muscle tissues were homogenized and the samples of tissue extracts were obtained by the centrifugation (16000 rpm, +4 °C).

The content of protein was determined with the technique developed by Greenberg [4]. In the tissue extracts the activity of antioxidant enzymes and the level of primary and secondary products of lipid peroxidation were evaluated.

The activity of glutathione peroxidase (GP) was determined using the method described in [28] and based on the determination of accumulated oxidized glutathione level.

The activity of glutathione reductase (GR) was evaluated in the reaction of reduction of oxidized glutathione [28] by the determining the rates of NADPH decrease.

The activity of total glutathione-S-transferase (GST) was assessed using the kit (“Sigma”, USA) in the reaction of reduced glutathione with 1-chloro-2, 4-dinitrobenzene by the measurement of rate of glutathione-S-2, 4-dinitrobenzene (GDNB) generation that is characterized with the absorbance maximum at 340 nm [14].

Activity of catalase (Cat) in tumors and muscle tissues was evaluated by the method [12] based on measurement of decrease of the complexes of ammonium molybdate and hydrogen peroxide.

The determination of lipid peroxidation (LP) byproducts. The level of diene conjugates (DC) was determined by the rate of appearance of new maximum (233 nm) in the absorbance spectrum of polyunsaturated fatty acids after the diene conjugation in the procedure described in [25]. The content of tiobarbiturate-

active products (TBAP) was evaluated accordingly to [26] in the reaction with tiobarbituric acid.

Statistical analysis. The results of investigations were analyzed using the common approaches of variation statistics. The level of significance of the differences between the groups was evaluated by the use of non-parametric Mann — Whitney U-test ($p < 0.05$).

RESULTS

Glutathione-dependent activities in LLC and LLC/R9 tumors, in CMT and IMT of mice are represented in Tables 1, 2, respectively. The results represented on Fig. 1 point out the absence of significant changes in the activity of GP in CMT of mice with LLC/R9 tumors comparatively to the CMT of mice with LLC tumors and to IMT.

Table 1. Activity of antioxidant enzymes in control muscle tissues of mice with transplanted tumors LLC and LLC/R9 and in muscle tissue of intact mice

Object	Characteristics			
	Activity of GST, micromol GDNB/mg of protein per min	Activity of GP, micromol GSSG/mg of protein per min	Activity of Cat, U/mg of protein per min	Activity of GR, micromol NADPH/mg of protein per min
Control muscle tissue of mice with LLC	1204.1 ± 570.3*	97.8 ± 17.0	7.6 ± 1.4*	1.8 ± 0.8*
Control muscle tissue of mice with LLC/R9	1994.1 ± 656.8	71.7 ± 22.5	26.8 ± 3.9***	16.9 ± 11.3**
Muscle tissue of intact mice	3938.7 ± 672.2	80.5 ± 16.5	193.5 ± 15.5	23.8 ± 12.8

Note: *differences are significant in comparison with the values of characteristics for IMT ($p < 0.05$); **differences are significant in comparison with the values of characteristics for LLC ($p < 0.05$).

Table 2. Activity of antioxidant enzymes in LLC and LLC/R9 tumors and in muscle tissue of intact mice

Object	Characteristics			
	Activity of GST, micromol GDNB/mg of protein per min	Activity of GP, micromol GSSG/mg of protein per min	Activity of Cat, U/mg of protein per min	Activity of GR, micromol NADPH/mg of protein per min
LLC tumors	840.2 ± 153.3*	191.4 ± 28.2*	35.6 ± 15.9*	0.75 ± 0.3*
LLC/R9 tumors	2241.5 ± 656.0**	67.2 ± 22.0**	18.8 ± 3.7*	7.9 ± 2.6**
Muscle tissue of intact mice	3938.7 ± 672.2	80.5 ± 16.5	193.5 ± 15.5	23.8 ± 12.8

Note: *differences are significant in comparison with the values of characteristics for IMT ($p < 0.05$); **differences are significant in comparison with the values of characteristics for LLC ($p < 0.05$).

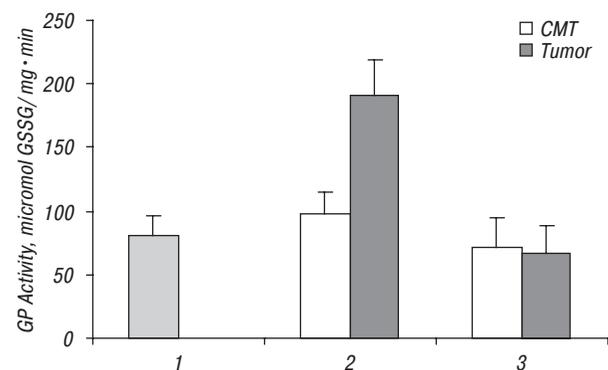


Fig. 1. The activity of glutathione peroxidase in tumors and control muscle tissue of tumor bearing mice (2 — LLC; 3 — LLC/R9) and in muscle tissue of intact mice (1)

However, the decrease in the value of this enzyme activity by 41% ($p < 0.05$) is observed in LLC/R9 tumors in contrast to LLC tumors (Table 2).

In contrary to GP, the level of Cat activity which is mostly localized in peroxisomes (but acts synergistically with GP towards transformation of hydrogen peroxide) was significantly decreased, as compared to IMT, in tumors of both types (original and modified variants of Lewis lung carcinoma) (Table 2). The level of Cat activity in CMT of LLC-bearing mice was equal to that of LLC tumors while the level of this enzyme activity was low, what was not characteristic for LLC/R9 tumors, compared to respective CMT (Table 1). Meanwhile Cat activity in the tumors didn't differ significantly along with observed tendency toward the decrease of this index in LLC/R9 tumors compared to LLC tumors (Table 2).

As an indicator of prooxidative processes intensity that is in relationship with the level of functioning of peroxidase chain in cells, the indexes reflecting LP intensity have been used. In LLC/R9 tumors and in CMT of these animals, a significant ($p < 0.05$) elevation of content of primary LP products compared to respective studied objects of mice with transplanted LLC cells has been observed, while the tissues didn't differ significantly by TBAP content (Fig. 2, 3). Comparative analysis of LP intensity indexes in tissues of mice with transplanted tumors and in IMT has evidenced on the increase ($p < 0.05$) of content of the products of diene conjugation of fatty acids in LLC/R9 tumors and in CMT of the mice, and on increase ($p < 0.05$) of content of secondary LP products in CMT of mice with LLC tumors compared with IMT, that could point on intensification of free radical oxidation of lipid components in organism of animals with transplanted tumors [1, 23].

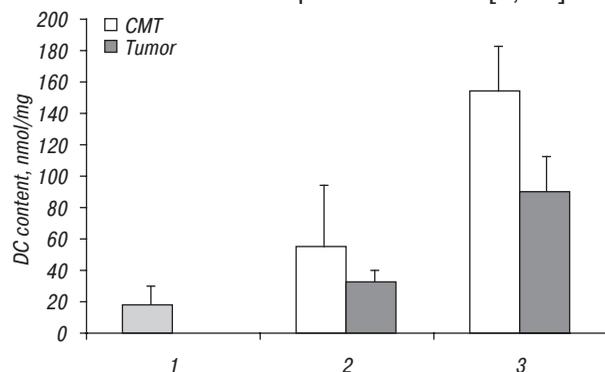


Fig. 2. The content of diene conjugates in tumors and control muscle tissues of tumor bearing mice (2 — LLC; 3 — LLC/R9) and in muscle tissue of intact mice (1)

In the studied tissues of mice with transplanted tumors the decrease of GP level compared to IMT, has been detected. However, the enzyme activity in LLC/R9 tumors and in CMT of the mice compared to respective tissues of mice with transplanted LLC cells, was significantly ($p < 0.05$) increased and nearly reached the activity level in IMT (Table 1, Table 2). An activity of total pool of glutathione-S-transferase in CMT of all tumor-bearing animals didn't differ, and was decreased compared with

its activity in IMT (Fig. 4). In LLC/R9 tumors, GST activity was elevated ($p < 0.05$) compared to the tumors of original variant LLC.

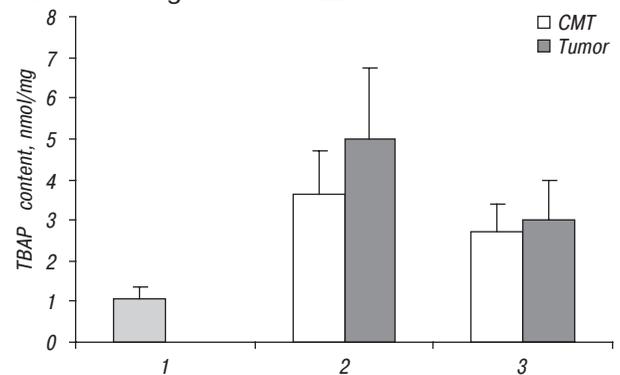


Fig. 3. The content of tiobarbiturate-active products in tumors and control muscle tissues of tumor bearing mice (2 — LLC; 3 — LLC/R9) and in muscle tissue of intact mice (1)

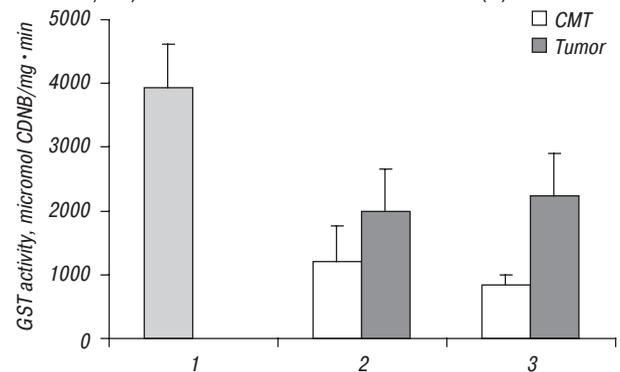


Fig. 4. The activity of total glutathione-S-transferase in tumors and control muscle tissue of tumor bearing mice (2 — LLC; 3 — LLC/R9) and in muscle tissue of intact mice (1)

DISCUSSION

The scheme presented on Fig. 5 reflects the relation and contingency of functioning of glutathione-dependent enzymes, catalase, and superoxide dismutase for maintenance of prooxidant-antioxidant balance of cell. The scheme demonstrates that for enzymatic activity, glutathione peroxidase and glutathione-S-transferase use the same substrate — reduced glutathione (GSH), the pool of which is renewed via activity of NADPH-dependent glutathione reductase. Due to the action of glutathione peroxidase, that is a key component of deactivation of cytotoxic hydroperoxides, the oxidation of reduced glutathione occurs, and this process is accompanied by reduction of cytotoxic peroxide compounds, while at the II-nd stage of cell detoxication glutathione-S-transferase utilizes reduced glutathione in the reactions of conjugation of xenobiotics of exogenous and endogenous origin metabolized on endoplasmic reticulum membranes with the involvement of cytochrome P-450.

Taking into account the contingency of functioning of glutathione-dependent — GP and GST that use the same substrate — reduced glutathione, and also GR, with the involvement of which the pool of the latest is replenished (Fig. 5), the revealed differences between the indexes of enzymatic activities of glutathione

system in LLC/R9 tumors and in CMT of the mice and respective tissues of mice with LLC tumors (Table 1, 2), evidence on more effective functioning of GST in LLC/R9 tumors; at the II-nd stage of detoxication GST is able to provide conjugation of xenobiotics, including medicinal means with the use of reduced glutathione, therefore providing the formation of mechanisms of drug resistance [27]. In CMT of mice with transplanted LLC/R9 tumors reduced GSH is used more effectively for oxidation of hydroperoxides of fatty acids, and hydrogen peroxide with the involvement of GP. Higher level of functional activity of GP in LLC/R9 tumors compared to studied tissues of mice with LLC tumors, evidences on effective renewal of oxidized glutathione form in tissues of LLC/R9 mice and supplement of substrate pool of GP and GST [15].

SUPEROXIDE DISMUTASE

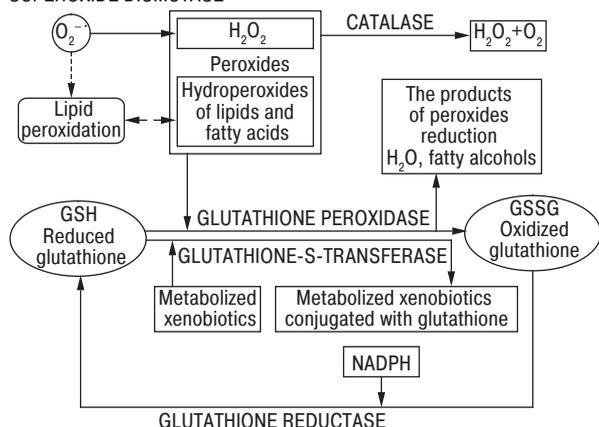


Fig. 5. The interrelation of functional activity of the key antioxidant enzymes

The presented results may be explained, among other reasons, by biological properties of LLC/R9 cell subline generated by experimental progression of LLC in the direction of formation of the resistance to cis-dichlorodiaminoplatine. Possibly, the formation of drug resistance in LLC/R9 tumors may be a consequence of induction of GST isoform that differs (in particular, by substrate specificity) from the molecular form expressed constitutively in tissues of intact animals. Also, it is possible that in tumors and in tissues of tumor-bearing mice the suppression of expression/activity of constitutively expressed form may occur as well [31]. The decrease or sometimes stable level of GST activity in CMT of mice with transplanted LLC tumors compared to IMT of mice could be explained by manifestation of tumor influence on organism.

The decrease ($p < 0.05$) of GP activity level in LLC/R9 tumors compared with LLC tumors (Fig. 1), may occur via depletion of reaction substrate — GSH, and also potential impact of accumulated ROS into the process of oxidative modification of active site and its allosteric regulatory centers could not be excluded [16], and this is in accordance with significantly higher DC level (Fig. 2) in CMT of mice with LLC/R9 tumors and in LLC/R9 tumors. However, elevation of functional activity of GP excludes the possibility of suppression of its activity via the shift of GSH/GSSG ratio in cell toward increased content of oxidized GSSG form that

couldn't be used effectively by enzyme. GP activity in CMT preparations of mice with LLC/R9 tumors didn't differ significantly from the intensity of this index in IMT.

However, in LLC/R9 tumors GP activity was by 41% lower ($p < 0.05$) than that in LLC tumors (Table 1). This could point on the fact that utilization of reduced glutathione in LLC tumors occurs at larger extent for detoxification of endogenous peroxides by glutathione peroxidase, what in turn may lead to lower sensitivity of LLC cells to metabolic stress.

The decrease of Cat activity in mice with LLC tumors and in tumors of both variants compared to IMT (Table 1, 2) evidences on depletion of the enzyme activity in tissue of mice with LLC tumors; among possible reasons, oxidative modification of enzyme active site could be considered [16], what is in accordance with higher level of LP intensity (Fig. 3). Higher level of Cat activity ($p < 0.05$) that is a characteristic for LLC tumors in comparison with respective CMT has not been detected in LLC/R9 tumors in comparison with the respective CMT. Taking into account the data on more expressed level of autophagy-associated death for LLC/R9 tumor cells *in vitro* compared to wild-type LLC cells, one may suppose that the compensatory increase of Cat activity ($p < 0.05$), that has been observed in LLC tumors versus respective CMT, has been leveled down in LLC/R9 tumors by processes of selective Cat degradation, a key initial step of autophagy [22, 29]. The registered decrease of Cat activity is in accordance with the data of other authors who have detected such patterns: the drop of the enzyme activity by 75–90% in malignant transformed keratinocytes correlated with manifestations of malignant phenotype of these cells [19]; negative transcription regulation of catalase gene in human hepatoma cells has been revealed, and this may point on possible role of regulatory mechanisms on transcription level [20]; Cat activity is significantly decreased in liver tissue of tumor-bearing rats, but after tumor removal returns to normal level [5].

The detected increase ($p < 0.05$) of DC content in CMT and LLC/R9 tumors versus CMT of mice with LLC tumors evidences on intensified primary LP reactions compared with secondary ones, due to elevation of the quantity of radicals able initiate conversion of fatty acids in the content of membrane lipids in LLC/R9 tumors.

As far as we have studied the tumors developed after transplantation of LLC and LLC/R9 variants — the cells of the same tissue origin and genotype, but differing by a number of phenotypic patterns, it seems to be correct to establish a relation between antioxidant enzyme activity and biological properties of the tumors.

So, the tumors developed after transplantation of wild-type strain of Lewis lung carcinoma that are characterized by tolerance for metabolic stress and high metastatic potential [18, 24], are also characterized by higher level of functioning of peroxidase enzymes (GP and Cat) at the background of higher LP intensity, associated with accumulation of secondary lipid peroxidation products, but lower levels of GST and GR activities in comparison with LLC/R9 tumors.

The sensitivity of LLC/R9 cells to deficiency of nutrient substrates is correlated with high GST activity level, higher GR activity, and higher intensity of primary LP processes. The latest is related to the lower volume of pool of free radical molecules able initiate chain reactions of oxidation at the background of lower level of functional activity of peroxidase enzymes.

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