Exp Oncol 2014
36, 1, 29–33

Experimental ONCOLOGY

DYNAMICS OF CHANGES OF ANTIOXIDANT SYSTEM INDEXES DURING THE GROWTH OF TWO LEWIS LUNG CARCINOMA VARIANTS

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Aim: **To study an intensity of prooxidant processes and activity of antioxidant enzymes in tumor tissue of two Lewis lung carcinoma variants (LLC and LLC/R9) differing by their proliferative, metastatic, and angiogenic potential (LLC/R9 as compared to LLC is characterized by lower metastatic activity, higher proliferative and angiogenic potential).** ***Materials and Methods*****: The *in vitro* study was carried out using cultured LLC and LLC/R9 cell lines, and *in vivo* on 50 female C57/BL6 mice. The indexes of prooxidant processes and activity of antioxidant enzymes have been studied using the methods of experimental oncology, optical spectroscopy, fluorescent spectroscopy, electron paramagnetic resonance, statistical analysis.** ***Results:*** **There has been determined the coherence of results on 1.5 fold higher ($p < 0.01$) level of spontaneous generation of reactive oxygen species (ROS) by LLC cells *in vitro* compared to LLC/R9 cells, and twice ($p < 0.05$) higher content of secondary products of lipid peroxidation at 14th and 17th day of tumor growth in LLC compared to that in LLC/R9. It has been shown that deficiency of nutrient substrates determines an increase ($p < 0.01$) of ROS production in LLC/R9 cells what is in congruence with the data on accumulation of nitrosyl complexes of heme iron in mitochondria of LLC/R9 during tumor growth. Activity of superoxide dismutase during tumor growth has altered in unmonotonous way: starting from 14th day of growth it sharply increased by 147% (17th day, LLC) and 217% (20th day, LLC/R9), and then decreased to the level registered at 14th day. Progressive decrease of activity of glutathione peroxidase (GP) and glutathione-S-transferase (GST) during LLC growth has been accompanied with the decrease of the level of reduced glutathione (GSH) by 70% ($p < 0.05$). In the case of LLC/R9 the decrease of GP activity at initial stages of tumor growth correlated with significant increase of GSH level in the tumor — by 250% ($p < 0.01$). It has been shown that in LLC/R9 tumors (unlike to LLC), GSH utilization is mostly provided by GST, its significantly higher activity has been detected in LLC/R9 tumors compared to LLC.** ***Conclusion:*** **We have revealed a number of peculiarities of antioxidant system functioning in LLC and LLC/R9 tumors and have shown a relation between an activity of antioxidant system and some biological properties of studied tumor variants.**

Key Words: **Lewis lung carcinoma variants, reactive oxygen species, lipid peroxidation, antioxidant enzymes, glutathione-dependent enzymes.**

Insufficient rate of vascularization compared with high proliferation rate of tumor cells (TC) results in limited supply of TC with oxygen and nutrients, accumulation of metabolic products and reactive oxygen species (ROS) in tumor microenvironment what could be characterized as a situation of metabolic stress [1]. From other side, hypoxic conditions promote manifestation of many effects of adaptive character directed toward TC survival, including suppression of apoptosis, increased glycolysis intensity, induction of autophagy and mitophagy, digestion of alternative energetic substrates, increased synthesis of angiogenesis factors etc [2, 3].

ROS occupy a central place in formation of adaptive response toward metabolic stress; their role as messengers in signal pathways involved in proliferation, TC death via apoptosis, necrosis or autophagy, regulation of cell energetic, angiogenesis, migration ability, interaction with extracellular stroma etc, is well-known [3]. At the same time an excess of ROS which could be caused by dysfunction of TC mitochondria as well as persistent inflammatory process in tumor lesion, determines the development of oxidative stress, damaging effects toward biologic membranes, proteins, lipids, nucleic acids. Along with this oxidative stress could be caused by malfunction of antioxidant system (AOS).

Realization of ROS effects in cell depends on AOS state which provides neutralization of free radical molecules and consists from high molecular weight enzyme components and low molecular weight non-enzyme components [4]. AOS activity determines preservation of TC viability in unfavorable conditions. Key ROS scavengers include superoxide dismutase (SOD) which neutralizes superoxide-anion into hydrogen peroxide which in turn is utilized by catalase (CAT) and glutathione peroxidase (GP) [4, 5]. The results of studies *in vitro* and *in vivo* point on relation between SOD functioning in tumors and their growth characteristics [6–8]. According to the data of other authors, some GP isoforms have membrane localization, while CAT which exerts neutralization of lipid peroxides, fatty acids, alkylperoxides, and hydrogen peroxide in locations close to cell membranes [9]. Also, intracellular redox status depends on ratio between reduced (GSH) and oxidized (GSSG) forms of glutathione, what is regulated with the involvement of GP, glutathione reductase (GR), and glutathione-S-transferase (GST) [5]. GST uses GSH as a substrate and participates in neutralization of xenobiotics metabolized with involvement of cytochrome P450, products generated

Received: September 24, 2013.
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Abbreviations used: AOS – antioxidant system; CAT – catalase; cis-DDP (cis-diamminedichloroplatinum); EPR – electron paramagnetic resonance; FBS – fetal bovine serum; GP – glutathione peroxidase; GR – glutathione reductase; GSH – reduced glutathione; GSSG – oxidized glutathione; GST – glutathione-S-transferase; iNOS – inducible NO-synthase; LPO – lipid peroxidation; mNOS – mitochondrial NO-synthase; NCHI – nitrosyl complexes of heme iron; ROS – reactive oxygen species; SOD – superoxidedismutase; TBARS – thiobarbituric acid reactive substances; TC – tumor cells.

via oxidative stress, endogenous toxic compounds, etc. [5, 10]. There has been shown a role of AOS components (in particular, Mn-SOD, peroxy- and thioredoxines, CAT) as proteins — tumor suppressors [6–8].

Evaluation of the state of AOS enzymes in TC differing by their biological patterns, could be informative for determination of the ways of TC adaptation to the deficiency of oxygen and nutrient substrates and also for identification of targets for possible influence on the state of oxidative processes in TC for induction of metabolic catastrophe, achievement of anticancer and antimetastatic effects. That's why this work was aimed on the study of intensity of prooxidant processes and activity of antioxidant enzymes in two Lewis lung carcinoma variants — LLC and LLC/R9 which differ by growth kinetics, intensity of metastasis, ability to autophagy, sensitivity to the action of inhibitors of tumor angiogenesis, and to deficiency of energetic substrates [11–14]. In particular, LLC/R9 as compared to LLC is characterized by lower metastatic activity, higher proliferative and angiogenic potential and significant sensitivity to antiangiogenic therapy and nutrient deficiency.

MATERIALS AND METHODS

In the study there were used two variants of Lewis lung carcinoma (LLC and LLC/R9) kindly provided by National Bank of Cell Lines and Transplanted Tumors of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine (Kyiv, Ukraine). LLC/R9 variant was generated from the wild-type LLC strain after 9 sequential cis-diamminedichloroplatinum (cis-DDP) cycles *in vivo* [11, 12]. The LLC and LLC/R9 cells were cultured *in vitro* in RPMI 1640 medium (Sigma, USA) supplemented with 10% fetal calf serum (Sigma, USA), 2 mM L-glutamine, and 40 μg/ml gentamicin at 37 °C in atmosphere with 5% CO_2. *In vivo* research has been performed on 50 C57/BL6 female mice 2–2.5 months old, weighing 18–23 g bred in vivarium of IEPOR NASU. The mice were inoculated with LLC and LLC/R9 cells in the thigh muscle of the right hind leg ($1 \cdot 10^6$ cells in 0.1 ml of Hank's solution).

The mice from 1st group (n = 25) were transplanted with LLC cells, the animals from 2nd group (n = 25) — with LLC/R9 cells. The study was approved by regional Ethics Committee and responded to the rules on the work with experimental animals.

Activities of antioxidant enzymes and LPO product content in tumor tissue homogenates were investigated during tumor growth at 14th, 17th, 20th, and 25th day after cancer cell inoculation (5–7 animals per each time point).

Total protein content in tumor tissue homogenates was determined by the method of Greenberg [15]. Total SOD activity was analyzed using spectrophotometry and measurement of nitroformazan content generated in reaction of superoxide-anion and nitroblue tetrazolium which competes with the enzyme [16]. CAT activity in the samples was determined according to [17] by the content of complex of ammonium molybdate and residual hydrogen peroxide. The content of thiobarbituric acid reactive substances (TBARS), secondary lipid peroxidation (LPO) products, was determined as described in [18] in the reaction with thiobarbituric acid. GP activity was analyzed by the method [19], based on determination of GSSG accumulation. GR activity was evaluated according to [19] by the decrease of NADPH content in the reaction of GSSG reduction. Activity of GST was determined with the use of kit [19] (Sigma, USA) by measuring the rate of generation of glutathione-S-2,4-dinitrobenzene in the reaction of GSSG and 1-chloro-2,4- dinitrobenzene [10]. Content of GSH was determined according to [20] in the reaction with 5,5'-dithiobis-2-nitrobenzoic acid.

Functional state of TC mitochondria was analyzed by the level of nitrosyl complexes of heme iron (NCHI) (NO-complexes) with $g_{av} = 2{,}007$ according to the data of EPR spectra. For this purpose, from tumor and muscle tissues there have been prepared cylindrical samples of special size ($d = 4.0$ mm, $l = 25–35$ mm), which were frozen and stored at temperature 77 K. EPR spectra were done at temperature 77 K using spectrophotometer E-109 Varian (USA). Muscle tissue from left leg of each mouse served as an individual control. To determine initial values of EPR signals, intact mice (n = 5) were used.

The level of intracellular ROS in TC was determined with the use of 2,7-dichlorofluorescein diacetate (Sigma, USA) after 1-day incubation of TC in the medium with different content of glucose and glutamine according to [21]. To create the deficiency in glucose and glutamine, we have used glucose-free medium (Sigma, USA) and glutamine-free medium (Sigma, USA) supplemented with 10% FBS (Sigma, USA) and gentamicin. Control cultures were incubated in standard medium which contains 11 mM glucose and 2 mM glutamine, and was supplemented with FBS and gentamicin.

Statistical analysis of obtained results was performed with the use of descriptive methods, and Student's *t*-criterion.

RESULTS

Intensity of prooxidant processes in LLC and LLC/R9 tumors. The results of the study of ROS production by LLC and LLC/R9 cells *in vitro* indicated significantly higher (by 60%, $p < 0.01$) level of spontaneous generation of free radical oxygen forms in LLC cells compared to LLC/R9 cells in the conditions of their cultivation in the medium with standard content of glucose and glutamine and upon normoxia (Fig. 1). Significant decrease of concentration of glucose or glutamine in culture medium did not shift significantly ROS level in LLC cells but caused an increase of ROS level in LLC/R9 cells by 26% ($p < 0.01$) and 76% ($p < 0.01$) respectively compared to the control.

The data on higher ROS level in LLC cells compared to LLC/R9 cells *in vitro* are in agreement with the results of the study of content of TBARS — secondary products of LPO. TBARS content in LLC tumors was by 50% higher than that in LLC/R9 tumors ($p < 0.05$), at least at 14th and 17th day after tumor transplantation (Fig. 2). The maximal elevation of TBARS level in LLC tumors was recorded at 17th day of growth, while in LLC/R9 tumors TBARS content did not differ significantly during tumor growth.

The data on the content of NCHI the presence of which evidences on disturbance of electron-transport com-

plexes of tumor mitochondrial respiratory chain, for both tumor variants in their growth dynamics are presented in Fig. 3 and show the different character of NCHI changes in the case of LLC and LLC/R9. In the case of LLC NCHI level remained relatively constant during tumor growth, while in the case of LLC/R9 the progressive increase of NCHI content has been recorded: by 163% ($p < 0.05$) and by 255% ($p < 0.05$) at 20th and 25th day respectively. At 25th day after tumor transplantation, the NCHI content in LLC/R9 tumors was by 178% higher than that in LLC tumors ($p < 0.05$).

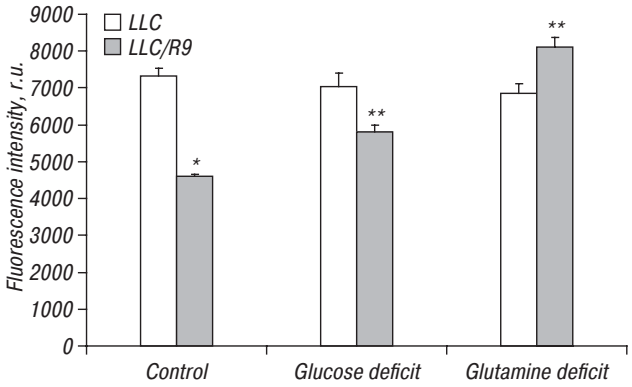

Fig. 1. Intracellular ROS level in LLC and LLC/R9 cells after 1 day of incubation in medium with different content of glucose and glutamine. *Differences are significant as compared to the corresponding value for LLC; **for LLC/R9 cells differences are significant as compared to the corresponding control ($p < 0.05$); r.u. — relative units

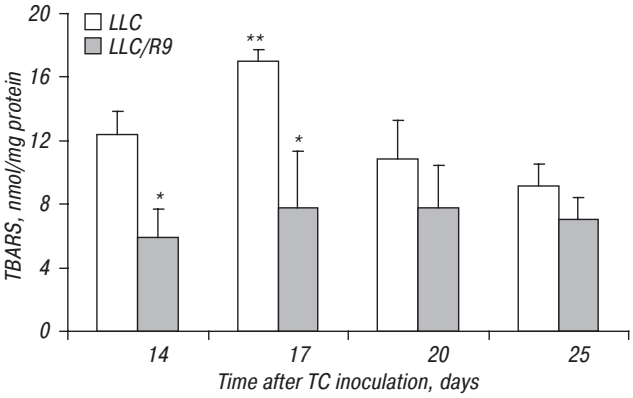

Fig. 2. The content of TBARS in tumor during LLC and LLC/R9 growth. *Differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day of LLC growth ($p < 0.05$)

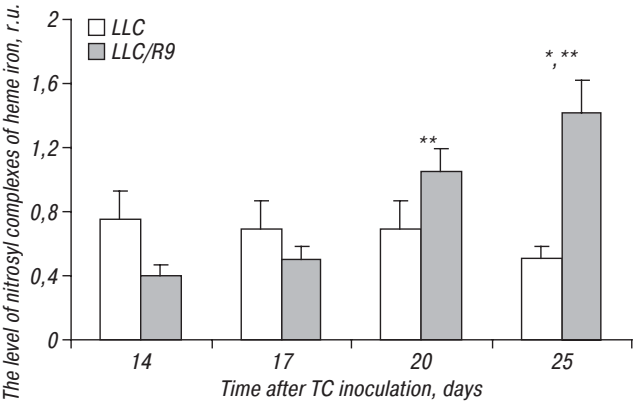

Fig. 3. The level of nitrosyl complexes of heme iron in tumor during LLC and LLC/R9 growth. *Differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day of LLC/R9 growth ($p < 0.05$); r.u. — relative units

Activities of SOD, CAT, and glutathione-dependent enzymes in LLC and LLC/R9 tumors. The results of study of the changes of SOD and CAT activities in LLC and LLC/R9 tumors during their growth are presented in Table 1. SOD activity in tumor tissue increased at the period of intense tumor growth in both tumor variants, however, in the case of LLC maximal SOD activity has been registered at 17th day of tumor growth, while in the case of LLC/R9 — at 20th day. Nonmonotonous character of CAT activity changes (at least of their mean values) was revealed for both LLC and LLC/R9. Trend of decrease of this activity was registered at 17th day of tumor growth but due to high variability of the values observed changes were not statistically significant.

Table 1. SOD and CAT activity in tumor during LLC and LLC/R9 growth

Time after TC transplantation, day	SOD, r.u./mg protein·min		CAT, r.u./mg protein·min	
	LLC	LLC/R9	LLC	LLC/R9
14	27.7±5.2	22.4±5.0	42.7±15.4	21.5±5.5
17	68.2±0.96**	34.6±0.6*	15.4±1.98	11.8±5.3
20	25.9±3.4	69.9±16.0*,**	41.8±8.6	28.9±4.5
25	27.0±7.2	21.2±5.6	23.9±3.5	21.4±2.3

Note: *differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day for each tumor ($p < 0.05$); r.u. – relative units.

The data on activities of glutathione-dependent enzymes (GP, GR, GST) in LLC and LLC/R9 tumors, are presented in Fig. 4 and Table 2. As it's seen in Fig. 4, GP activity in tumor tissue of both tumor variants has been decreasing monotonically. For example, in both tumor variants GP activity has decreased from its maximal values registered at 14th day of tumor growth by more that 70% ($p < 0.05$) at 20th day.

Table 2. GST and GR activity in tumor during LLC and LLC/R9 growth

Time after TC inoculation, day	GST, μmol CDNB/ mg protein·min		GR, μmol NADPH/ mg protein·min	
	LLC	LLC/R9	LLC	LLC/R9
14	1.142±0.165	2.138±0.311*	12.95±1.50	7.30±2.90
17	0.411±0.207**	1.444±0.207*	7.42±2.72	9.99±1.15
20	0.678±0.079**	1.916±0.372*	9.51±1.02	4.40±0.98*
25	0.732±0.103	1.385±0.462	6.13±1.36**	N/A

Note: *differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day for each tumor ($p < 0.05$); N/A – not available.

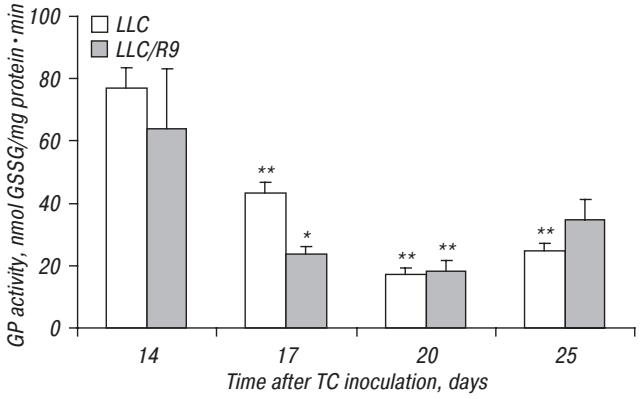

Fig. 4. GP activity in tumor during LLC and LLC/R9 growth. *Differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day for each tumor ($p < 0.05$)

Unlike to GP, the character of changes in GST activity during LLC growth significantly differed from that

of LLC/R9. As it is shown in Table 2, in the case of LLC GST activity in tumor homogenates has been decreasing significantly along with tumor growth: in particular, at 17th and 20th days its activity was lower by 64% ($p < 0.05$) and by 41% ($p < 0.05$) compared with respective index at 14th day. At the same time in the case of LLC/R9, GST activity in tumor homogenates was 2–4 fold higher ($p < 0.05$) than respective indexes in case of LLC and didn't change significantly during tumor growth.

As it seen in Table 2 LLC and LLC/R9 growth was accompanied by the decrease of GR activity. The reduction of GR level by 40% ($p < 0.05$) was observed at 20th day of LLC/R9 growth. In case of LLC statistically significant decrease of GR activity by 53% was shown at 25th day as compared to that at 14th day of tumor growth.

The decrease of GP and GST activities in tumor tissue during LLC growth was not accompanied by expected increase of reduced glutathione (GSH), which is a substrate for these enzymes. In contrary, at 20th and 25th days of LLC growth GSH content in tumor decreased by 70% ($p < 0.05$) and 62% ($p < 0.05$) compared to corresponding value at 14th day respectively (Fig. 5).

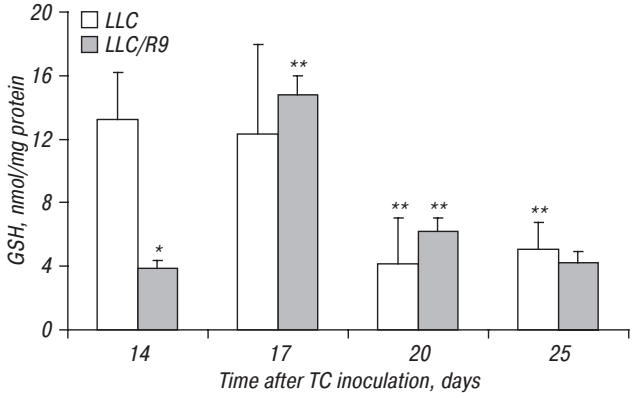

Fig. 5. The content of reduced glutathione (GSH) in tumor during LLC and LLC/R9 growth. *Differences are significant as compared to the corresponding value for LLC; **differences are significant as compared to the value on the 14th day for each tumor ($p < 0.05$)

In the case of LLC/R9, GSH content at 14th day of tumor growth was by 3.5 fold lower ($p < 0.05$) compared to respective value in the case of LLC and increased (4 fold, $p < 0.05$) up to maximal values at 17th day whereupon GSH level significantly ($p < 0.05$) diminished at 20th and 25th days.

DISCUSSION

Despite their identical origin and genotype, two variants of Lewis lung carcinoma — LLC and LLC/R9 significantly differ by their biologic properties. LLC/R9, obtained by experimental progression of parental variant LLC toward formation of drug resistance to cis-DDP, is characterized by low metastatic potential and high proliferation potential compared to LLC [11, 12, 22]. For example, at 25th day of tumor growth the number of lung metastases and their volume in mice with transplanted LLC/R9 is nearly 3 times lower compared to these indexes in animals with LLC, while the volume of primary tumors in mice with LLC/R9 is nearly twice larger than that in LLC-bearing mice. LLC/R9 is characterized also by high (compared to LLC) angiogenic potential which is expressed in 1.5 times higher VEGF production level (as key inducer of tumor angiogenesis), high sensitivity to inhibitors of tumor angiogenesis and significant dependence of TC growth from nutrient substrate content.

It is known that during tumor development its microenvironment significantly alters toward an increased deficiency of nutrient substrates (energetic and plastic ones) and accumulation of metabolic products of tumor and normal cells (involved in tumor process) [23]. Such changes in TC microenvironment appear due to deficiency of tumor tissue vasculature (caused by pathologic angiogenesis), leading to formation of metabolic stress, altered tumor cellular composition, and biologic patterns of the cells. Significant role in such changes belongs to prooxidant processes and peculiarities of functioning of antioxidant system of TC [1–3]. The results of our study support these observations.

The study of AOS functioning upon LLC and LLC/R9 growth has shown that the character of alteration of activity of some enzymes of antioxidant defense and intensity of prooxidant processes in tumor tissue differed between these tumor variants. An intensity of prooxidant processes was evaluated by the content of secondary LPO products, NCHI and ROS levels in TC. LLC, highly-metastatic variant, was characterized by increased content of LPO secondary products (at least at intense period of tumor growth) compared to LLC/R9, and significantly higher basal ROS level (mostly peroxides) *in vitro* compared to LLC/R9 cells under conditions of cell incubation in culture medium with standard content of glucose and glutamine.

At the same time in the case of LLC/R9 nutrient substrate deficiency (which is usually observed in a growing tumor) led to statistically significant increase of ROS content, what could be possibly related to dysfunction of TC mitochondria and/or insufficient functioning of their antioxidant system. The first assumption has been supported with our data on NCHI accumulation in TC mitochondria and decreased content of iron-sulfur centers (data not shown) during LLC/R9 growth. As it is known, these complexes are formed via production of nitrogen radicals (NO) by mitochondrial NO-synthase (mNOS) in TC and/or inducible NO-synthase (iNOS) in macrophages, and are capable to modulate the rate of electron transport through the components of electron-transport chain of mitochondria and initiate cell hypoxia. In such conditions NO may react with superoxide-radical with following generation of peroxynitrite — an oxidizing and nitrosylizing agent, and cause the development of mitochondrial dysfunction [24]. Mitochondrial dysfunction of LLC/R9 cells may also develop due to higher (compared to LLC) sensitivity to nutrient substrate deficiency; in LLC/R9 cells which were incubated for a long period without replacement of culture medium and were deficient in nutrient substrates, there has been recorded an appearance of structurally defective mitochondria [13].

Some differences between LLC and LLC/R9 have been also found in the character of kinetics of changes in activity of antioxidant enzymes, in particular, SOD — first-line antioxidant defense enzyme. Despite the fact that maximal SOD value in both carcinoma variants was practically equal, in the case of LLC its maximal activity was achieved at 17th day of tumor growth while in the case of LLC/R9 —

only at 20th day. It should be noted that maximal level of SOD is observed at 17th day of LLC growth when these cancer cells are characterized by maximum migration capacity resulting in their high metastatic activity [11, 12]. The relation between SOD activity and metastatic potential of a tumor is supported by the data [25, 26]. According to the data of literature, an excess of H_2O_2, which appears due to increased SOD activity and decreased GP activity (which were observed at 17th day of LLC growth *versus* same indexes at 14th day), could determine an initiation of signal cascades which positively regulate migration/metastatic activity of TC, in particular, activation of Snail, increase of the content of phosphorylated ERK1/2 kinase, activation of matrix metalloproteinase-9, cause structural instability of E-cadherin, etc [25]. From other side, an excess of H_2O_2, may also determine oxidation of polyunsaturated fatty acids with generation of alkylperoxides and fatty acid peroxides leading to increased accumulation of LPO products which has been observed at 17th day of LLC growth compared to that at 14th day.

The functioning of glutathione-dependent system during LLC growth also differed from that in LLC/R9, in particular by the character of changes in GSH content and GST activity. Really, the obtained data evidence on the fact that in LLC/R9 tumors GSH utilization is mostly directed on support of functional activity of GST as an enzyme which has a higher affinity to substrate; the level of GST activity was significantly higher in LLC/R9 tumors compared to LLC. Usually, glutathione level and activity of enzymes of glutathione-dependent system in drug-resistant malignant cells are increased [27, 28]. It could not be excluded that significantly higher level of GST activity, peculiarities of alterations in GSH content in LLC/R9 during tumor growth and low ROS level in LLC/R9 cells could be related to drug resistance as far as LLC/R9 variant was generated by multistage experimental progression of the wild-type LLC strain toward formation of the resistance to cis-DDP.

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