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State of the antioxidant system in mitochondria of skin cells during experimental B16/F10 melanoma growth with chronic neurogenic pain

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ABSTRACT

Aim. To study the state of the antioxidant system in mitochondria of skin cells during B16/F10 melanoma growth in mice with chronic neurogenic pain.

Materials and methods. The study included female C57BL/6 mice (n = 28). Experimental groups included an intact group, a control group – chronic neurogenic pain model, a comparison group – standard subcutaneous transplantation of B16/F10 melanoma, and a main group – transplantation of B16/F10 melanoma 3 weeks after creation of a model of chronic neurogenic pain. Animals were decapitated on day 14 of the B16/F10 melanoma growth, the skin was excised and mitochondria were isolated. Standard ELISA test systems were used to determine the levels of reduced glutathione (GSH) and oxidized glutathione (GSSG) (Bio Source, USA); glutathione peroxidase-4 (GPx 4) (Clod-Clon Corporation, CNDR); glutathione reductase (GR) (Cusabio, CNDR); glutathione S-transferase (G-S-T) (Ivvundiagnostik, FRG); glutathione peroxidase-1 (GPx 1), and superoxide dismutase-2 (SOD-2) (Ab Frontier, South Korea).

Results. Mitochondria of skin cells in controls showed an increase in the levels of GSH by 1.3 times, GPx 1 – by 2.9 times, GPx 4 – by 1.9 times, GR – by 2.8 times, and SOD-2 – by 2.4 times, compared to intact animals. Changes in the comparison group were opposite: GPx 1 decreased by 1.9 times, GPx 4 – by 3.7 times, GR – by 3.9 times, SOD-2 – by 3.8 times, and GSSG rose by 1.36 times compared to intact animals. The growth of melanoma with chronic neurogenic pain caused an increase in the levels of GSH by 1.5 times, GPx 1 – by 3.6 times, G-S-T – by 1.28 times, GPx 4 – by 1.6 times, and SOD-2 – by 1.8 times, compared to intact animals.

Conclusions. The growth of B16/F10 melanoma together with chronic neurogenic pain restructures the antioxidant system of skin mitochondria towards generation of reductive stress under the influence of chronic pain, which can affect the growth and development of experimental melanoma.

Key words: experimental B16/F10 melanoma, chronic neurogenic pain, skin, antioxidant system, mitochondria.

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Conformity with the principles of ethics. The animal studies were conducted in compliance with humanity principles set forth in the Directive of the European Union (86/609/EEC) and the Declaration of Helsinki. The study was approved at the session of the Bioethics Committee for Working with Animals of Rostov Research Institute of Oncology (Protocol No. 4 dated 10/08/2018). All participants of the study signed an informed consent to participate in the research.

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Состояние антиоксидантной системы в митохондриях клеток кожи при росте экспериментальной меланомы B16/F10 на фоне хронической нейрогенной боли

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РЕЗЮМЕ

Цель – изучить состояние антиоксидантной системы в митохондриях клеток кожи при росте меланомы B16/F10 у мышей на фоне хронической нейрогенной боли.

Материалы и методы. Работа выполнена на самках мышей линии C57BL/6 (n = 28). Экспериментальные группы: интактная, контрольная – воспроизведение модели хронической нейрогенной боли, группа сравнения – стандартная подкожная перевивка меланомы B16/F10, основная группа – перевивка меланомы B16/F10 через 3 нед после создания модели хронической нейрогенной боли. Животных на 14-е сут роста меланомы B16/F10 декапитировали, иссекали кожу, выделяли митохондрии. Тест-системой для иммуноферментного анализа определяли уровень восстановленного глутатиона (GSH), окисленного глутатиона (GSSG) (Bio Source, США); глутатионпероксидазы-4 (ГПО-4) (Clod-Clon Corporation, CNDR); глутатионредуктазы (ГР) (Cusabio, CNDR); глутатион-S-трансферазы (ГТ) (Ivvundiagnostik, FRG); глутатионпероксидазы-1 (ГПО-1), супероксиддисмутазы-2 (СОД-2) (Ab Frontier, Южная Корея).

Результаты. В митохондриях клеток кожи в контрольной группе установлено повышение содержания GSH в 1,3 раза; ГПО-1 – 2,9; ГПО-4 – 1,9; ГР – 2,8; СОД-2 в 2,4 раза относительно значений у интактных животных. В группе сравнения обнаружили принципиально противоположные изменения: снижение содержания ГПО-1 в 1,9 раза; ГПО-4 – 3,7; ГР – 3,9; СОД-2 в 3,8 раза и повышение уровня GSSG в 1,36 раза по сравнению со значениями у интактных животных. При росте меланомы на фоне хронической нейрогенной боли отмечено увеличение уровня GSH в 1,5 раза; ГПО-1 – 3,6; ГТ – 1,28; ГПО-4 – 1,6 и СОД-2 в 1,8 раза по сравнению со значениями в интактной группе животных.

Заключение. При росте меланомы B16/F10 на фоне хронической нейрогенной боли происходит перестройка антиоксидантной системы митохондрий клеток кожи в сторону реализации «восстановительного стресса» под воздействием хронической боли, что может оказывать влияние на рост и развитие экспериментальной меланомы.

Ключевые слова: экспериментальная меланома B16/F10, хроническая нейрогенная боль, кожа, антиоксидантная система, митохондрии.

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INTRODUCTION

Skin melanoma is characterized by an extremely high degree of malignancy and an exceptionally high potential of lymphatic, hematogenous or lymphohematogenous spread. Traditionally, skin melanoma is considered to have a variable, frequently unpredictable clinical progression, including both cases of spontaneous involution of the primary tumor lesion and early spread of the neoplastic process with favorable prognostic signs available [1]. In recent years,

certain success has been achieved in understanding the etiology of this disease which is associated with the anatomic localization, the extent of exposure to ultraviolet radiation, genetic particularities, and, potentially, other factors, too [1, 2].

Any pathological process undergoes the initial change at the hyperfine level. The borderline of the transition from the norm to pathology requires an in-depth study at the atomic, molecular, and sub-cellular levels [3]. The ability to adapt cellular bioenergetic capacities under the influence of rapidly changing conditions of the environment is compulsory both in the normal cellular function and in the development of tumors [4]. It is mitochondria that are highly sensitive indicators of pathological processes emerging in the body. Being the central metabolic organelle, they perform crucial biochemical functions in synthesizing the main cellular components, including fatty acids, aminoacids, and nucleotides. Cells of many tumors containing completely functional mitochondria increase the speed of glycolysis for maintaining proliferation and survival, thus ensuring a higher flow of substrate for biosynthesis pathways partially performed in mitochondria (metabolism of glucose and lipogenesis, metabolism of aminoacids, and biosynthesis of nucleotides). As a result of the activation of the metabolic flow through mitochondrial pathways, the production of ROS in tumor cells increases, which entails activation of antioxidative response pathways of the cells [4].

The skin is a common target organ for melanoma, and it is a unique and the largest organ/tissue of the body accumulating numerous physiological functions. The skin is a standalone organ and the key interface between the endocrine, nervous and immune systems. The skin is an integral sophisticated tissue system incorporating several layers which are closely connected with one other. In the skin, circulatory, lymphatic and nervous pathways are present that enable it to quickly respond to pain, mechanic, chemical, thermal, and other stimulations by luminal narrowing or dilation with the subsequent change of the blood flow. The vascular tone (lumen of vessels and blood flow speed) of the skin is influenced by the cerebral cortex via numerous vasoconstrictor and vasodilator nerve endings, owing to which the body ability to feel warmth, cold, pressure, tactile sense, and pain is fulfilled [5]. Pain is frequently an accompanying component of a neoplastic process, and it is present in 30 – 50% of cancer patients after the performed anticancer therapy and in 65 – 90% of patients due to disease progression [6]. As a rule, the origin of pain in oncological patients is multi-factorial: it arises from direct and indirect effects of the tumor growth, a side effect of antineoplastic therapy, and concurrent diseases [6]. Experimental oncology moves towards understanding the biological and physiological processes arising in the body in combined concurrent chronic pain and tumor development. In particular, chronic neurogenic pain was reported to stimulate and modify the growth of cutaneous melanotic cancer in the experiment [7]. Therefore, studying antioxidative processes in mitochondria of skin cells under pathological processes accompanied by pain syndrome seems highly relevant. Certainly, pathophysiological processes can be studied by means of experimental models. It is indisputable that development of experimental oncology with an extensive study of pathophysiology of the malignant process using experimental animal models promotes advancement of both practical and theoretical oncology.

The aim of the research was to study the antioxidative system in mitochondria of skin cells in experimental animals with chronic neurogenic pain, tumor development, and the combined effect of these pathological processes.

MATERIALS AND METHODS

The study was performed using female C57BL/6 mice (n = 28) aged 8 weeks weighing initially 21–22 g. The animals were received from Scientific Center of Biomedical Technologies "Andreevka" (Moscow region). The cell culture of murine B16/F10 melanoma metastasizing into the lungs was used. The tumor strain was obtained from N.N. Blokhin National Medical Research Center of Oncology.

The animals were kept under natural lighting mode with free access to water and food. All studies were conducted in compliance with the requirements and conditions stated in the International Guiding Principles for Biomedical Research Involving Animals and the Order of the Russian Ministry of Health No. 267 dated 19/06/03 "On the approval of laboratory practice rules".

The animals were distributed by the random sampling method into the following experimental groups: the intact group (n = 7), the control group with reproduction of the chronic pain model [8] (n = 7), the comparison group (B16/F10) – mice with standard subcutaneous transplantation of melanoma B16/F10 (n = 7), and the main group (chronic pain + B16/F10) – the B16/F10 melanoma was grafted 3 weeks after cre-

ating the chronic pain model (n = 7). In the mice of the main group (chronic pain + B16/F10), bilateral ligation of sciatic nerves was performed under the xylo-zoletil anesthesia. 3 weeks after healing of the surgical wound, 0.5 ml of suspension of B16/F10 melanoma tumor cells in the physiological solution diluted at 1:10 was introduced subcutaneously under the right shoulder blade. B16/F10 melanoma was grafted in the animals of the comparison group (B16/F10) subcutaneously at the same dosage and volume as that in the main group, but without reproducing the chronic pain model. In standard transplantation, the tumor emerges in 100% of cases, grows quite rapidly and metastasizes on days 12–16 into the lungs (60–90%) mainly hematogenously, less frequently it spreads into the liver and spleen. All manipulations with animals were performed in a sterile cabinet. The tools, utensils, and hands were disinfected by the conventional method.

All animals were decapitated by guillotine on day 14 of the experiment. After decapitation, the animals' skin was quickly excised with application of cooling agents, and mitochondria were isolated using the method of M.V. Egorov and S.A. Afanasyev [9]. In the obtained mitochondrial samples, the following levels were identified using the standard ELISA test systems: reduced glutathione (GSH) in nM/g protein (Bio Source, USA), oxidized glutathione (GSSG) in nM/g protein (Bio Source, USA), glutathione peroxidase-1 (GPx 1) in ng/mg protein (Ab Frontier, South Korea), glutathione peroxidase-4 (GPx 4) in ng/mg protein (Cloud-Clone Corporation, DPRK), glutathione reductase (GR) in ng/mg protein (Cusabio, DPRK), glutathione S-transferase (G-S-T) in ng/mg protein (Immundiagnostik, FRG), superoxide dismutase-2 (SOD-2) in pg/mg protein (Ab Frontier, South Korea), and total protein by the biuret method, g/l (Olveks Diagnosticum, Russia).

The statistical analysis of the results was performed using the Statistica 6.0 software package. The quantitative data for four groups (independent samples) were compared using the Kruskal – Wallis test with multiple comparison. The data are presented in the form of $M \pm m$, where M is the arithmetic mean value, and m is the standard error of the mean.

RESULTS

During the experiment, data were obtained on the influence of B16/ F10 melanoma, chronic neurogenic pain, and the combined effect of these pathological processes on the glutathione system in mitochondria of skin cells in female mice on the 14th day of the experiment – the logarithmic phase of the experimental tumor growth (Table 1). Higher concentrations of antioxidative system components were registered in mitochondria of skin cells in the animals with chronic neurogenic pain (the control group), as compared to the intact animals: GSH increased by 1.3 times, GPx 1 – by 2.9 times, GPx 4 – by 1.9 times, GR – by 2.8 times, and SOD-2 – by 2.4 times.

At the same time, malignant growth in animals of the comparison group led to completely opposite changes in the antioxidative system in the mitochondria: the content of GPx 1 decreased by 1.9 times, GPx 4 – by 3.7 times, GR – by 3.9 times, SOD-2 – by 3.8 times,

Table 1

Levels of antioxidant enzymes in mitochondria of skin cells in female mice during the growth of B16/F10 melanoma with chronic neurogenic pain

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
Reduced glutathione (GSH) (nM/g protein)	151 071.2 ± 717.41	196 860.1 ± 1 917.581 p^1= 0.000000	179 886.5 ± 15 147.22	219 446.3[1] ± 3 643.97 p^1 = 0.00000
Oxidized glutathione (GSSG) (nM/g protein)	462.32 ± 15.88	506.81 ± 19.83	637.25 ± 17.62[1,2] p^1= 0.000008 p^2 = 0.000356	714.34 ± 9.16[1,2] p^1 = 0.00000 p^2 = 0.000001
GPx 1 (ng/mg protein)	0.207 ± 0.009	0.604 ± 0.007[1] p^1 = 0.00000	0.107 ± 0.008[1,2] p^1 = 0.00005 p^2 = 0.00000	0.746 ± 0.014[1,2,3] p^1 = 0.00000 p^2 = 0.00001 p^3 = 0.00000
GPx 4 (ng/mg protein)	16.518 ± 0.216	32.263 ± 0.471[1] p^1 = 0.00000	4.435 ± 0.166[1,2] p^1 = 0.00000 p^2 = 0.00000	25.903 ± 0.282[1,2,3] p^1 = 0.00000 p^2 = 0.00000 p^3 = 0.00000

Table 1 (continued)

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
GR (ng/mg protein)	13.239 ± 0.190	36.717 ± 0.228[1] $p^1 = 0.00000$	3.403 ± 0.222[1,2] $p^1 = 0.00000$ $p^2 = 0.00000$	16.675 ± 0.189[2,3] $p^2 = 0.00000$ $p^3 = 0.00000$
Glutathione S-transferase (G-S-T) (ng/mg protein)	2.164 ± 0.127	1.898 ± 0.100	1.771 ± 0.099	2.433 ± 0.145[2,3] $p^2 = 0.010500$ $p^3 = 0.002727$
SOD-2 (pg/mg protein)	461.402 ± 20.133	1 128.2 ± 54.186[1] $p^1 = 0.00000$	120.84 ± 10.904[1,2] $p^1 = 0.00000$ $p^2 = 0.00000$	849.68 ± 32.492[1,2,3] $p^1 = 0.00000$ $p^2 = 0.00085$ $p^3 = 0.00000$

Note. 1 – statistically significant value compared to the values in intact animals; 2 – statistically significant value compared to the values in controls (chronic pain); 3 – statistically significant value compared to the values in the comparison group (B16/F10 tumor).

and GSSG rose by 1.36 times compared to the intact values. The same trend remained during comparison with the control animal group (chronic neurogenic pain). Here, the level of GPx 1 dropped by 5.6 times, GR – by 10.8 times, GPx 4 – by 7.3 times, and SOD-2 – by 9.3 times. The content of GSSG exceeded the control group values by 1.26 times. Meanwhile, GSH levels did not differ significantly from the intact and control values.

The combined effect of chronic neurogenic pain and tumor process contributed to an increase in production of GSH by 1.5 times, GPx 1 – by 3.6 times, G-S-T – by 1.28 times, GPx 4 – by 1.6 times, and SOD-2 – by 1.8 times, as compared to the values for the intact animal group. The content of GSSG was 1.54 times higher than the intact figures. As compared to the values in the animals suffering from chronic neurogenic pain only, the combined effect of two pathological processes exhibited 1.24 times higher level of GPx 1 ($p = 0.00001$), 2.2 times higher level of GR, 1.37 times higher level of G-S-T (at the level of the statistical trend), while SOD-2 and GPx 4 were 1.33 times and 1.24 times lower, respectively ($p = 0.00000$). The level of GSSG was 1.42 times higher, too. The comparison of a combination of chronic pain and tumor growth with tumor growth only (the comparison group) demonstrated a rise in GPx 1 by 6.9 times, GR – by 4.9 times, G-S-T – by 1.37 times, GPx 4 – by 5.8 times, and SOD-2 – by 7 times, respectively.

Table 2 presents ratios of the glutathione system components reflecting the maintenance of redox homeostasis in the animal organism.

The dominating inhibition of antioxidative components in tumor development and their excess in chronic pain introduced differently directed shifts into the function of physiological cascades of antioxidant enzymes. In particular, control animals (chronic neurogenic pain) demonstrated 1.22 times increase in the GSH/GSSG ratio and 2.2 times decrease in the GSH/GPx 1 ratio, as compared to the intact values. Tumor growth in the comparison group contributed to the reduction of the calculated ratios as follows: SOD-2/GPx 1 was 2 times lower, GR/GPx 1 – 2 times lower,

Table 2

Parameters of the glutathione cascade in mitochondria of skin cells in female mice during the growth of B16/F10 melanoma with chronic neurogenic pain

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
GSH/GSSG	3.2 ± 0.124	3.9 ± 0.130[1] $p^1 = 0.004849$	2.8 ± 0.300[2] $p^2 = 0.007518$	3.1 ± 0.053[2] $p^2 = 0.000064$
GSH/GPx 1	7.3 ± 0.298	3.3 ± 0.052[1] $p^1 = 0.000000$	17.8 ± 2.567[1,2] $p^1 = 0.001583$ $p^2 = 0.000102$	2.94 ± 0.039[1,2,3] $p^1 = 0.000000$ $p^3 = 0.000084$

Table 2 (continued)

Parameters	Intact animals	Control animals (chronic neurogenic pain)	Comparison group (melanoma B16/F10)	Main group (chronic neurogenic pain + melanoma B16/F10)
SOD-2/GPx 1	2.2 ± 0.129	1.8 ± 0.100	$1.1 \pm 0.112^{1,2}$ $p^1 = 0.000036$ $p^2 = 0.000464$	$1.1 \pm 0.052^{1,2}$ $p^1 = 0.000004$ $p^2 = 0.000031$
GR/GPx 1	6.4 ± 0.258	6.0 ± 0.115	$3.2 \pm 0.324^{1,2}$ $p^1 = 0.000006$ $p^2 = 0.000003$	$2.2 \pm 0.063^{1,2,3}$ $p^1 = 0.000000$ $p^2 = 0.000000$ $p^3 = 0.008103$

Note. 1 – statistically significant value compared to the values in intact animals; 2 – statistically significant value compared to the values in controls (chronic pain); 3 – statistically significant value compared to the values in the comparison group (B16/F10 tumor).

while the GSH/GPx 1 ratio, by contrast, significantly increased by 2.44 times. As compared to the group with chronic neurogenic pain, the ratios in the group with tumor growth were reduced: the GSH/GSSG ratio fell by 1.4 times (p = 0.007518), SOD-2/GPx 1 – by 1.64 times, GR/GPx 1– by 1.87 times, while the GSH/GPx 1 ratio rose by 5.4 times. The combined effect of two pathological processes inhibited the function of physiological cascades of antioxidant enzymes. So, GSH/GPx 1 decreased by 2.5 times as compared to the intact values, SOD-2/GPx 1 – by 2 times and GR/GPx 1 – by 2.9 times, respectively. In comparison with the control group of animals (chronic neurogenic pain), changes were observed for the GSH/GSSG, SOD-2/GPx 1, and GR/GPx 1 ratios, which manifested themselves through the reduction of their values by 1.26 times (p = 0.000064), 1.64 times, and 2.7 times, respectively. The decrease in GSH/GPx 1 by 6 times and GR/GPx 1 – by 1.45 times was found, compared to the group of animals with tumor growth (the comparison group). Changes in the cascade reactions were associated with GPx 1 responsible for mechanical detoxification of peroxides performed with the help of the enzymatic bi-bi mechanism with two molecules of GSH. Due to this, enzymatic detoxification of non-radical hydroperoxides took place, and the oxidative-reductive balance was regulated directly by elimination of hydroperoxides and oxidation of GSH, the main low-molecular thiol in the cells [10]. The overabundance of GPx 1 is quite likely to result in a lack of potential substrates, which manifests itself through inhibition of all cascades with its participation.

DISCUSSION

Analyzing the obtained data, the authors believe that mitochondria of skin cells in the presence of chronic neurogenic pain respond towards the reductive stress, which manifests itself in the active production of all the enzymes studied, including the tripeptide – GSH. In the case of the neoplastic process in the skin mitochondria – the target tissue for melanoma – the classical scenario of the peroxide theory of carcinogenesis is observed [11] with inhibition of all antioxidant enzymes and accumulation of oxidized glutathione. Chronic neurogenic pain in animals with the tumor process prevents melanoma from changing the reductive stress that already developed. Due to this, all antioxidant enzymes are at quite high levels, but the enzymatic cascade interaction is inhibited, which is demonstrated by the values of the calculated ratios: GSH/GPx 1, GR/GPx 1. The excessive accumulation of reductive equivalents leads to reductive stress; it is also characterized by the absence of oxidants and/or by the decrease in excessive equivalents [12, 13]. The concept of reductive stress is rather new. For a certain time, the absence of cellular oxidants has been known to reduce the growth responses of cells. Newer pieces of evidence point to additional cellular and physiological effects caused by the absence of cellular oxidants and accumulation of excessive reductive equivalents, including changes in the formation of disulphide protein bond, reduced mitochondrial function, and lower cellular metabolism [13]. At present, a number of studies confirm that the “deoxidizing”, or reductive, stress accompanies such conditions as hypoxia and hyperglycemia, that inhibit the mitochondrial function and cause the excessive accumulation of cellular reducing equivalents [14–16]. The authors believe that chronic neurogenic pain belongs to the same conditions which can change the function of mitochondria (in this case, mitochondria of skin cells) towards reductive stress, and the trend remains if the neoplastic process joins. The increasing amount of antioxidant

enzymes under the effect of chronic neurogenic pain at the moment of tumor appearance may be at such a stable condition which cannot be reverted or inhibited by the oxidative process which, in its turn, characterizes the tumor growth.

CONCLUSION

The obtained results showed that chronic neurogenic pain has a modulating effect on the functioning of skin cell mitochondria, promoting a shift of their antioxidant system towards the reductive stress, which manifests itself through an essential increase in the content of antioxidant enzymes. Such a response of mitochondria of skin cells can result in other patterns in the development of the neoplastic process during chronic neurogenic pain. Definitely, it is only skin that is in question, and not the entire organism on the whole, and the authors realize that a functional response of mitochondria in different organs can vary. This is what causes interest and requires further research.

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Neskubina I.V. – conception and design; analysis and interpretation of data. Frantsiyants E. M. – conception and design; final approval of the manuscript for publication. Surikova E.I. – analysis and interpretation of data. Kaplieva I.V. – conception and design. Trepitaki L.K. – conception and design. Nemashkalova L.A. – substantiation of the manuscript, critical revision for important intellectual content. Lesovaya N.S. – substantiation of the manuscript, critical revision for important intellectual content.

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