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EFFECT OF ADHESIVE LLC CELL PRETREATMENT BY OXAMATE ON THE SURVIVAL INDEXES AFTER TRANSITION TO DE-ADHESIVE GROWTH

Background. The ability to metabolic reprogramming is a distinctive feature of metastatically active tumor cells. A classic example of metabolic reprogramming, characteristic of almost all malignant cells, is aerobic glycolysis. Therefore, inhibition of glycolysis in tumor cells is considered a promising strategy for antitumor therapy. **Aim.** To generate Lewis lung carcinoma (LLC) cell subpopulation after pretreatment by a lactate dehydrogenase (LDH) inhibitor — oxamate in adhesive growth conditions, and then to study the metabolism of this subpopulation in the anchorage-independent growth conditions. **Materials and Methods.** LLC cells were cultured without oxamate or with 17 mM oxamate in the adhesive growth conditions with the following transition to the anchorage-independent growth conditions without oxamate. A distribution of LLC cells by cell cycle phases, apoptosis rate, levels of reactive oxygen species (ROS), E-cadherin, and vimentin were determined by flow cytometry. Glucose consumption and lactate production were determined by spectrophotometry. **Results.** 48-h oxamate treatment in adhesive growth conditions resulted in a 30% decrease of the total number of LLC cells compared to the control. In 72 h after the transfer of both oxamate-treated and control cells into the anchorage-independent growth condition without oxamate, the number of viable cells pretreated with oxamate was reduced by 17% ($p < 0.05$) compared to the control cells. However, the distribution of cells by cell cycle phases did not differ. In cells pre-treated with oxamate, the rate of glucose consumption decreased by 20% ($p < 0.05$), ROS generation was reduced by 17%, vimentin expression decreased by 10% while the rate of lactate production was the same in oxamate-pretreated and control cells. **Conclusion**. The cytostatic effect of oxamate demonstrated in adhesive growth conditions persisted for 72 h in the anchorage-independent growth conditions. The absence of differences in the cell cycle phase distribution and a decrease in the ROS generation may indicate the initial stage of overcoming the cytostatic effect of oxamate after 72 h of culturing LLC cells in anchorage-independent growth conditions.

Keywords: oxamate, glycolysis, lactate dehydrogenase, reactive oxygen species, anchorage-independent growth, glucose consumption, lactate production, vimentin.

The metastatic cascade has an extremely complex and dynamic nature covering the entire body, from the primary tumor to the metastases in distant organs and tissues [1, 2]. Each stage of this cascade depends on many factors which are often not related to each other. The ability to metabolic reprogramming is now recognized as a distinctive feature of the metastatically active tumor cells [3].

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A classic example of metabolic reprogramming is aerobic glycolysis characteristic of almost all malignant cells, which causes an increase in the rate of glycolysis and lactate production even in the presence of oxygen (the so-called "Warburg effect") [4, 5]. Such a metabolic shift provides survival advantages for tumor cells, as they can produce energy and maintain a high level of anabolic processes even under short or long-term hypoxia. Moreover, aerobic glycolysis can guarantee a high rate of synthesis of intermediate products, which are necessary as building materials for actively proliferating cells. The intensification of lactate production, in turn, significantly affects the tumor microenvironment, causing remodeling of the extracellular matrix and contributing to the invasion and metastasis of malignant cells [6]. Therefore, inhibition of glycolysis in tumor cells is considered a promising strategy for antitumor therapy [7, 8].

The enzyme lactate dehydrogenase (LDH) isoforms LDHA and LDGB belong to the class of oxidoreductases [9, 10]. LDHA is the main enzyme for maintaining aerobic glycolysis, which supports the regeneration of nicotinic amide dinucleotide (NAD+) in the reaction of converting pyruvate into lactate [11]. An increased expression of LDHA correlates with intense malignant cell growth, large tumor volumes, activation of metastasis, immunosuppression, and resistance to radiation and chemotherapy [12—16]. LDHA inhibitors reduce the tumor growth rate and lactate concentration in the tumor microenvironment, which leads to a decrease in its acidity and activation of the immune killer cells [17, 18]. On the other hand, LDHA blockers with different mechanisms of action, such as oxamate, dichloroacetate, and aminooxyacetate can cause side effects in the human body due to their nonspecificity [19]. The studies aimed at elucidating the toxicity of LDH blockers have shown that deficiency of the LDH enzyme has little effect on the human body. Patients taking LDH blockers feel discomfort that is not life-threatening and is associated with such symptoms as muscle weakness, fatigue, and myoglobinuria [20]. Therefore, the targeted use of LDH blockers could be considered a potentially safe therapeutic strategy.

The anticancer effect of oxamate has been convincingly demonstrated in colorectal cancer cells [21], gastric cancer [22], medulloblastoma [23], glioma [24], pancreatic [25], and non-small cell lung cancer cells [26]. However, numerous studies have focused primarily on the effects of oxamate in *in vitro* models of adhesive cell growth or solid tumors. The influence of oxamate on cancer cell dissemination has been poorly studied so far. There are no data on the use of oxamate as neoadjuvant therapy and its influence on cancer cells in free circulation (i.e., potentially metastatic cells).

In the present study, we examined the action of oxamate, an esoteric form of pyruvate, a competitive inhibitor of LDHA activity. We have used oxamate during cell culturing in adherent conditions as an *in vitro* model of neoadjuvant therapy for a solid tumor. The growth of cells *in vitro* in non-adherent conditions served as a model of anchorage-independent growth.

The study was aimed at generating Lewis lung carcinoma (LLC) cell subpopulation pretreated with oxamate, which inhibited LDH in the conditions of adhesive growth followed by further investigation of some characteristics of this subpopulation in the conditions of anchorage-independent growth.

Materials and Methods

Cell lines. Lewis lung carcinoma cells (LLC) were obtained from the National Bank of Cell Lines and Tumor Strains of the IEPOR NASU. LLC cells were maintained *in vitro* in RPMI-1640 nutrient medium (Biowest, France) supplemented with 10% fetal calf serum (Biowest, France) and penicillin-streptomycin (Biowest, France) at 37 °C in humid conditions with 5% CO_2.

Counting viable LLC cells. The number of cells and their viability were estimated by a routine method using a 0.4% solution of trypan blue and a hemocytometer.

Anchorage-independent growth of cancer cells. To prevent the adhesion of LLC cells to the substrate during their incubation, the surface of Petri dishes was treated with Poly(2-hydroxyethyl methacrylate) polymer (PolyHEMA, Sigma–Aldrich, USA). PolyHEMA was dissolved in 96% ethyl alcohol at a concentration of 8 mg/mL, and 2 mL was applied to 60-mm dishes, then dried at 41 °C for 10—15 days until the alcohol completely evaporated. Before planting tumor cells in Petri dishes coated with polyHEMA, their surface was washed twice with 2 mL of phosphate-buffered saline (PBS).

Evaluation of the cytotoxic/cytostatic effect of oxamate on LLC cells in conditions of adhesive growth. To evaluate the cytotoxic/cytostatic effect of oxamate *in vitro*, 3 million LLC cells in 7 mL of RPMI-1640 medium were seeded in 100-mm dishes (without polyHEMA) and incubated overnight under standard conditions. After pre-incubation, the medium containing 17 mM oxamate (Sigma–Aldrich, USA) was added, and cells were further incubated for 48 h. The cells incubated under the same conditions with the addition of fresh nutrient medium without oxamate served as a control. After 48 h of incubation with oxamate, the number of viable cells was estimated using crystal violet staining (Sigma–Aldrich, USA) and a StatFax 2100 spectrophotometer (Awareness Technology, USA) at a wavelength of 595 nm.

Distribution of cells by cell cycle phases and the level of apoptosis were estimated by flow cytofluorometry. The cells were resuspended in a hypotonic lysis buffer (0.1% sodium citrate, 0.1% Triton X-100), which contained 5 μg/mL of propidium iodide and RNase, incubated for 40 min at 37 °C, and washed with PBS. DNA content was analyzed using a flow cytometer (Navios EX Beckman Coulter, USA) at an argon laser wavelength of 488 nm. The number of apoptotic cells was estimated by the sub-G0/G1 peak. The distribution of cells by the cell cycle phases was determined by mathematical modeling using the ModFit program.

Evaluation of the rate of glucose consumption. The concentration of glucose in the samples of the incubation medium was estimated using an automatic biochemical analyzer ChemWell 2910 (Awareness Technology, USA) and a commercial kit for the determination of glucose content according to the manufacturer's instructions. Samples were frozen as soon as possible at –20 °C for further storage. The glucose consumption rate (GCR) was estimated by the level of glucose in the incubation medium and the number of cells in a certain period according to the formula:

$$GCR = 2 \times \frac{(C_{gl}(t_i)) - (C_{gl}(t_{i+1})) \times V}{N(t_i) + N(t_{i+1})}$$

where $Cgl(t_i)$, $Cgl(t_{i+1})$, $C_L(t_i)$, $C_L(t_{i+1})$ are the concentrations of glucose in the incubation medium at t_i and t_{i+1} (two consecutive days of cell growth), $N(t_i)$ and $N(t_{i+1})$ are the counts of cells on the corresponding consecutive days of growth; V is the volume of the incubation medium which in the study was equal to 6×10^{-3} L.

Evaluation of the rate of lactate production. The level of lactate in the samples was determined on a biochemical analyzer using a commercial kit for the determination of lactate according to the manufacturer's instructions. For this, samples of the incubation medium were frozen as soon as possible at –20 °C for further storage.

The lactate production rate (LPR) was calculated by the level of lactate in the incubation medium and the number of cells in a certain period according to the formula:

$$LPR = 2 \times \frac{(C_L(t_{i+1})) - (C_L(t_i)) \times V}{N(t_i) + N(t_{i+1})}$$

where $C_L(t_i)$, $C_L(t_{i+1})$ are the concentrations of lactate in the incubation medium at t_i and t_{i+1} — two consecutive days of cell growth, $N(t_i)$ and $N(t_{i+1})$ are the counts of cells on corresponding consecutive days of growth; V is the volume of the incubation medium which in the study was equal to 6×10^{-3} L.

Expression level of E-cadherin and vimentin. The expression of E-cadherin was determined using Anti-CD324 (Alexa Fluor™ 488) Clone DECMA1 (Invitrogen, USA), and vimentin was determined using Rb mAb to Vimentin (EPR 3776) (Alexa Fluor™ 647) (Abcam, UK). LLC cells were removed from the Petri dishes by the Versene solution (Vetline Agroscience, Ukraine), washed twice with PBS and resuspended in 0.1 mL of PBS with added labeled antibodies, incubated for 30 min, washed with buffer, and analyzed on a flow cytometer.

Activity of production of reactive oxygen species (ROS). Determination of the activity of ROS production in tumor cells was carried out using staining by 2′,7′-dichlorofluorescein diacetate (Sigma-Aldrich, USA). LLC cells after the anchorage-independent growth were washed twice with 5 mL of PBS, resuspended in 0.5 mL of a 20 μM solution of 2′,7′-dichlorofluorescein diacetate in PBS, and incubated for 40 min at 37 °C in a CO_2 incubator. After staining, the cells were washed twice with PBS. Then samples were analyzed by flow cytometry. The percentage of stained cells (i.e., those producing ROS) and the intensity

of their fluorescence (i.e., the content of intracellular ROS) were determined.

Statistical methods. Statistical analysis of the data was performed using descriptive statistics, Student's *t*-test and Mann — Whitney U-test, and linear and non-linear regression analysis with the use of Microsoft Excel (Microsoft, USA) and Origin 2020 (OriginLab, USA) software.

Results and Discussion

The cells before replacement in anchorage-independent growth were pretreated with 17 mM oxamate for 48 h in the adhesive growth conditions. After that, the survival and level of cell apoptosis as well as GCR and LPR were determined in the control and experimental groups. After 48-h incubation in adhesive growth, the cells were planted on Petri dishes covered with polyHEMA (for modeling an anchorage-independent growth) and incubated for 72 h. The survival and level of cell apoptosis, GCR and LPR, LDH activity, levels of ROS generation, distribution by the cell cycle phases, and the expression of E-cadherin and vimentin were determined in both growth conditions.

Metabolism of LLC cells during incubation with oxamate in adhesive growth conditions. The data showed that in adhesive growth conditions, the total number of cells and the number of viable cells after incubation with oxamate significantly decreased by 30% and 29%, respectively, compared to the control (Table 1). The number of dead cells accounted for 5.88% and 4.38% of the total number of cells in the control and experimental group, respectively. The percentage of apoptotic cells was also insignificantly different between the groups (6.7% in the control *vs*. 7.8% in the experimental group). Therefore, in the given incubation conditions, 17 mM oxamate exerts a cytostatic effect on LLC cells. This effect could be explained by the fact that LDH is an enzyme that selectively catalyzes the reduction of pyruvate to L-lactate in combination with the oxidation of NADH to NAD+. A decrease in the concentration of NAD+ in the cytosol inhibits its glycolysis and, as a result, suppresses cancer cell proliferation.

The rate of glucose consumption was found to be different between the groups. The addition of oxamate significantly reduced the rate of glucose consumption by 20% and the rate of lactate production by 14% (Table 2). Based on the stochastic ratio of lactate/glucose, two molecules of lactate can be formed from one molecule of glucose, and the conversion factor will be equal to 2. In this case, all the glucose entered the cell will be processed into lactate without supplying glucose to the pentose phosphate pathway and the formation of intermediates

Table 1. **Survival of LLC cells incubated with or without oxamate in adhesive growth conditions**

Group	Number of cells ($\times 10^6$)		
	Total	Viable	Dead
Control	12.69 ± 0.82	11.95 ± 0.73	0.74 ± 0.23
Experimental	8.91 ± 0.76*	8.52 ± 0.59*	0.39 ± 0.18*

Note: * n = 4, *difference is statistically significant ($p < 0.05$) compared to the control.

Table 2. **Metabolic parameters of LLC cells incubated with or without oxamate in adhesive growth conditions**

Group	Metabolic parameter	
	Glucose consumption, μmol / (10^6 viable cells × h)	Lactate production, μmol / (10^6 viable cells × h)
Control	0.39 ± 0.01	0.48 ± 0.01
Experimental	0.31 ± 0.01*	0.41 ± 0.01*

Note: * n = 4, *difference is statistically significant ($p < 0.05$) compared to the control.

Table 3. **Survival of LLC cells in anchorage-independent growth conditions after pre-incubation with oxamate in adhesive growth conditions**

Group	Number of cells ($\times 10^6$)			
	Total	Viable	Dead	Apoptosis
Control	3.48 ± 0.18	3.26 ± 0.16	6.35 ± 0.38	10.47 ± 0.78
Experimental	2.88 ± 0.16*	2.71 ± 0.14*	5.90 ± 0.28	11.25 ± 0.82

Note: * n = 4, *difference is statistically significant ($p < 0.05$) compared to the control.

necessary for proliferation. In our experiments, the coefficient of conversion of glucose to lactate was 1.23 and 1.34 in the control and experimental groups, respectively. This result evidences a decrease in the efficiency of aerobic glycolysis associated with a decrease in the use of glucose for the synthesis of glycolytic intermediates necessary for proliferation.

Metabolism of LLC cells in conditions of anchorage-independent growth after preincubation with oxamate. After incubation of LLC cells with oxamate in adhesive growth conditions, the cells were removed and seeded into polyHEMA-coated dishes. The parameters of survival and metabolic plasticity were determined on the 3rd day of anchorage-independent growth. The data showed that after 72 h of growth in the experimental group (pre-incubated with oxamate in adhesive growth conditions), the total number of cells and the number of viable cells were significantly lower (by 17%) compared to the control group (Table 3). The number of dead cells in the control and experimental groups was equal to 6.35% and 5.91% of the total number of cells, respectively. The percentage of apoptotic cells did not differ significantly between the groups and did not exceed 11%. Since there was no significant difference in the number of dead cells and cells in a state of apoptosis, and the rate of cell proliferation in the experimental group was lower, it can be assumed that the cytostatic effect of oxamate persisted under anchorage-independent growth conditions even after its absence in the incubation medium.

In the cell cycle investigation, no differences were detected between two groups in the cell cycle phase distribution (Table 4).

A high rate of glucose consumption is characteristic of cancer cells since the activation of aerobic glycolysis provides intermediates that are necessary for cell proliferation. The rates of glucose consumption and lactate production in LLC cells after 72 h of anchorage-independent growth did not differ significantly between the groups (Table 5). Also, the LDH activity in the experimental group did not differ from the activity in the control group. This fact additionally indicated that within 3 days after exposure to oxamate, cells practically restored their glycolytic metabolism.

We have revealed that 99% of cells produced ROS on the 3rd day of growth in anchorage-independent conditions in both groups. However, the intensity of ROS production in the cells of the experimental group significantly decreased by 17% compared to the control (Table 6). Since the rate of glucose consumption did not change compared to the control values, and the amount of ROS decreased, it can be assumed that this is due to the redistribution of glucose and its redirection toward the production of glutathione in the pentose phos-

Table 4. **Cell cycle distribution of LLC cells in anchorage-independent growth conditions after pre-incubation with oxamate in adhesive growth conditions**

Group	Distribution of cells by cell cycle phases, %		
	G1/G0	S	G2/M
Control	34.92 ± 0.14	48.58 ± 0.16	16.50 ± 0.08
Experimental	34.37 ± 0.17	48.51 ± 0.18	17.12 ± 0.09

Table 5. **Metabolic parameters of LLC cells in anchorage-independent growth conditions after pre-incubation with oxamate in adhesive growth conditions**

Group	Metabolic parameter		
	Glucose consumption, μmol / (10^6 viable cells × h)	Lactate production, μmol / (10^6 viable cells × h)	LDH activity, U/10^6 viable cells
Control	0.33 ± 0.01	0.56 ± 0.01	10.40 ± 0.57
Experimental	0.34 ± 0.01	0.55 ± 0.01	10.94 ± 1.31

Table 6. **ROS production and expression of E-cadherin and vimentin in LLC cells in anchorage-independent growth conditions after pre-incubation with oxamate in adhesive growth conditions**

Group	Intensity of production, a.u.		
	ROS	E-cadherin	Vimentin
Control	197.80 ± 10.12	7.15 ± 0.71	75.50 ± 1.54
Experimental	161.80 ± 9.08*	8.61 ± 0.63	68.10 ± 2.13*

Note: * n = 4, *difference is statistically significant ($p < 0.05$) compared to the control.

phate pathway rather than the formation of glycolytic intermediates necessary for proliferation. This may also explain the persistent decrease in proliferation observed in non-adherent cells that were pretreated with oxamate.

Epithelial-mesenchymal transition (EMT) is thought to facilitate cancer cell migration and metastasis. It has been shown that LDH participates in the regulation of genes involved in the activation of EMT, in particular, Snail, Slug, E-cadherin, N-cadherin, vimentin, and fibronectin [27]. Our studies of E-cadherin expression by LLC cells did not reveal any difference. A decreased vimentin expression in the experimental group could indicate a reduction in the in the migration ability of LLC cells after incubation with oxamate, which persists after 72 h of incubation in anchorage-independent growth conditions. A decrease in mesenchymal marker expression can reduce cell motility and metastatic potential, which indicates the promise of using LDH inhibitors in cancer therapy.

In conclusion, our study has shown that after 72 h of culturing in anchorage-independent growth conditions, LLC cells are capable of overcoming the action of oxamate applied in doses equal to IC_{30}. One of the mechanisms of this phenomenon may be incomplete blockade of LDH activity, which provides the necessary glucose consumption and lactate production and, as a consequence, maintains the rate of aerobic glycolysis.

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ВПЛИВ ПОПЕРЕДНЬОЇ ОБРОБКИ ОКСАМАТОМ КЛІТИН LLC НА ПОКАЗНИКИ ВИЖИВАНОСТІ ПІСЛЯ ПЕРЕХОДУ ДО НЕЗАЛЕЖНОГО ВІД СУБСТРАТУ РОСТУ

Стан питання. Здатність до метаболічного перепрограмування є важливою особливістю метастатично активних пухлинних клітин. Класичним прикладом такого перепрограмування, яке є характерним для більшості злоякісних клітин, є аеробний гліколіз. Тому інгібування гліколізу у пухлинних клітинах вважається перспективною стратегією у протипухлинній терапії. **Мета**. Отримати субпопуляцію клітин LLC, яка проявляє стійкість до інгібування лактатдегідрогенази оксаматом при адгезивному рості, а також дослідити метаболізм цієї субпопуляції під час росту, незалежному від закріплення. **Матеріали та методи**. Визначення клітинного циклу, апоптозу, активних форм кисню, Е-кадгерину і віментину за допомогою проточної цитометрії. Оптична спектрофотометрія використовувалася для визначення споживання глюкози та продукування лактату. **Результати**. Дослідження впливу оксамату (17 мМ) на адгезивний ріст клітин LLC показали зменшення загальної кількості клітин на 30% через 48 год ($p < 0,05$) порівняно з контролем. Відсоток мертвих клітин не відрізнявся між двома групами. Отримані дані вказують на цитостатичну дію оксамату в дозі 17 мМ на клітини LLC при адгезивному рості. Потім клітини з придбаною стійкістю до дії оксамату при адгезивному рості були переміщені в умови незалежного від закріплення росту без оксамату. Дослідження виживаності цих клітин показало зменшення кількості живих клітин на 17% ($p < 0,05$) через 72 год порівняно з контролем. Не помічено відмінностей у розподілі клітин за фазами клітинного циклу між двома групами. Швидкість споживання глюкози в дослідній групі знизилася на 20 % ($p < 0,05$), а швидкість продукції лактату не відрізнялася від контрольних значень. Генерація активних форм кисню в дослідній групі була знижена на 17% ($p < 0,05$), а експресія віментину на 10% ($p < 0,05$) в порівнянні з контрольною. **Висновок**. Цитостатичний ефект оксамату, виявлений при адгезивному рості, продовжується 72 год в умовах росту незалежного від закріплення без додавання оксамату. Відсутність відмінностей у фазах клітинного циклу та зниження генерації активних форм кисню клітинами LLC може свідчити про початкову стадію подолання пухлинними клітинами цитостатичного впливу оксамату через 72 год.

Ключові слова: оксамат, гліколіз, лактатдегідрогеназа, активні форми кисню, незалежний від субстрату ріст, споживання глюкози, продукція лактату, віментин.