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ANALYSIS OF GROWTH KINETICS AND PROLIFERATIVE HETEROGENEITY OF LEWIS LUNG CARCINOMA CELLS GROWING AS UNFED CULTURE

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Aim: **To analyze the growth kinetics and proliferative heterogeneity of Lewis lung carcinoma (LLC) cells during their growth in monolayer for 5 days without replacement of culture medium (unfed culture).** ***Methods:*** **Cell biology methods, sandwich enzyme-linked immunosorbent assay for vascular endothelial growth factor (VEGF) detection (ELISA), enzymatic glucose-oxidase method for glucose measurements, mathematical modeling.** ***Results:*** **Created mathematical model showed good fit to experimental data; that allowed to determine kinetic (model) parameters of LLC cells and predict the changes in number of proliferating and quiescent cells (proliferative heterogeneity) during their growth. It was shown that growth kinetics of viable LLC cells possesses non-monotonous character — during first three days of growth the number of cells raised exponentially, with following decrease after the maximal level was achieved. At the same time the decrease of number of viable cells/increase of number of dead cells has been observed upon complete depletion of culture medium by glucose content. Glucose dependence of cell transition rate from proliferation to resting state predicted by mathematical model possessed a pronounced two-phase character. At a wide range of relatively high glucose concentrations (> 1.0 mg/ml) the transition rate was close to zero. At concentrations lower than 0.7 mg/ml, the rate of transition swiftly increased resulting in sharp change in cellular composition. At an interval from 70 to 90 h, practically all proliferating cells transited to a resting state. The rate of quiescent cell death was relatively low, and this was in part caused by too low level of glucose consumption compared to proliferating cells. It was shown that during LLC cells growth VEGF production rate decreased monotonously in spite of the fact that the level of VEGF in incubation medium increased monotonously. Observed monotonous decrease of VEGF production rate could not be explained by VEGF degradation in incubation medium (our results displayed the stability of VEGF molecule during investigations).** ***Conclusions:*** **Weak dependence of cell transition rate from proliferating to resting state from glucose level (> 0.7 mg/ml) and low rate of cell death provided slow decrease of the pool of quiescent cells in the population, thus significantly increasing their chance to survive upon nutritional deficiency.**

Key Words: **Lewis lung carcinoma, growth kinetics, proliferative heterogeneity, mathematical modeling.**

It is known that tumor cells could differ practically by all observed cell characteristics such as duration of mitotic cycle, migration ability and reaction on the action of endogenous and exogenous factors. Such variability could be observed also in cell populations derived from a single cell (clones), and one may ascribe it to heterogeneity of cell population or in more common sense to clonal heterogeneity [1, 2]. In all mentioned cases, heterogeneity is caused by existence of cell subpopulations with different properties (genotypic or phenotypic heterogeneity) as well as variability of each separate subpopulation composed from proliferating and quiescent cells (proliferative heterogeneity).

Numerous studies have shown that upon cultivation at equal conditions *in vitro*, cell population (or a clone) is characterized by great variability of growth kinetic curves [3, 4]. The analysis with the use of mathematical model has revealed that such phenomenon is caused by proliferative heterogeneity of population at initial stage of its growth, in particular, by the number of viable cells in the population and by the ratio between dividing and quiescent cells [5].

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Abbreviations used: ELISA – enzyme-linked immunosorbent assay; LLC – Lewis lung carcinoma; VEGF – vascular endothelial growth factor.

It is known also that microenvironment could influence significantly proliferative heterogeneity of tumor cell population and its growth kinetics. Deficiency of nutrient substrates (oxygen, glucose etc) could shift cell ratio toward elevated number of quiescent cells (G10 as well as G20) and decreased fraction of viable cells [6, 7]. Influence of mitogenic agents (growth factors etc.) may lead to elevation of the pool of actively proliferating cells and growth rate of the population. Cytoreductive antitumor preparations (cycle-specific and phase-specific ones) alter significantly composition of tumor cell population [8]. Moreover, efficacy of their action depends significantly on proliferative heterogeneity of tumor cells, because the majority of anticancer drugs possesses cycle-specific activity, i. e. are mainly effective against proliferating cells [9]. One should note that antiangiogenic therapy belongs to arsenal of cytoreductive anticancer therapy, and its goal is to reduce the pool of tumor cells via formation of metabolic stress (part of which is deficiency of nutrient substrates) by inhibition of tumor neovascularization.

Despite existence of numerous publications, the problem of control of growth of heterogeneous cell populations is far from being solved yet. At the same time the analysis of growth kinetics of cell population including characteristics of its proliferative heterogeneity could be useful for prediction of tumor progression and reaction of tumor to chemotherapy and

irradiation [10–12]. It's quite necessary to understand the mechanisms that underlay diversification of kinetic repertoire of tumor cells and providing their maximal survival. To answer this question one should reveal the characteristics of cell assembly that determine changes in growth kinetics and composition of cell population, and the only way to do it — is the application of mathematical model [13, 14].

That's why the aim of present work was analysis of growth kinetics and proliferative heterogeneity of Lewis lung carcinoma (LLC) cells during their growth in monolayer for 5 days without replacement of culture medium (unfed culture). One should note that the conditions of tumor cells cultivation in unfed culture that provide depletion of incubation medium from energetic and plastic substrates as well as accumulation of metabolic products of cells (elevation of lactate content, decrease of pH, accumulation of different growth factors produced by cells etc.) are modeling tumor cell microenvironment in the regions of tumor distant from blood vessels [4, 15]. Such regions are characteristic for malignant tumors and develop due to higher growth rate of tumor mass compared to that of new vessels, or as a result of application of antivascular or antiangiogenic therapy [16]. In this context, kinetic characteristics of cell growth in the frame of unfed culture model may reflect the degree of tumor cell sensitivity to antiangiogenic therapy. This point is important because there are no methods for prognosis of sensitivity of tumor to such therapy and evaluation of its efficacy [17], whilst high cost of antiangiogenic therapy and the necessity of its prolonged administration (as a rule, life-time one) dictates the necessity for the development of new preparations [18, 19].

MATERIALS AND METHODS

Cancer cell line and culture conditions. Lewis lung carcinoma (LLC, National Bank of Cell Lines and Cancer Strains, IEPOR) cells were used in this work. Stock culture was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS, Sigma), 2 mM L-glutamine and 40 μg/ml gentamycine at 37 °C in incubator with humidified atmosphere containing 5% CO_2.

All experiments were started from stock cultures maintained in exponential growth. The experiments were begun by seeding cells in 7 ml complete medium supplemented with 15% FBS in 6-cm tissue culture dishes. After 16 h of preincubation the culture medium was replaced with fresh one. This point was considered as starting time 0 h. After incubation at each time point, the cells were harvested by scraping all attached cells from culture dishes (with light microscope control) using a scraper, collected by centrifugation, stained with trypan blue and counted using a hemocytometer. Concurrently the number and viability of un-attached cells in supernatant were determined.

For "unfed" culture experiments the cells were incubated during 5 days (initial density of viable cells at starting time point was 0.15 ± 0.02 millions cells/dish). In case of comparative "unfed/fed" culture study the cells were incubated during 4 days (initial density of viable cells at starting time point was 0.24 ± 0.01 millions cells/dish) with the replacement of medium with the fresh one every day.

All the experiments were performed in a duplicate.

The level of glucose in culture media was determined by enzymatic glucose-oxidase method with using glucose assay kit (Sigma, cat. No GAGO-20) according to manufacturer's protocol. For that medium samples were collected, centrifuged at 1000 g and stored at –20 °C until glucose concentration was analyzed. Glucose concentration in complete culture medium samples were also measured before experiments in order to obtain starting values. The experiments were performed at a glucose concentration of 1.98 mg/ml (11 mM).

The level of VEGF was detected in cell culture supernatants in all above indicated points of time of the tumor cell growth by sandwich enzyme-linked immunosorbent assay (ELISA) with using mouse VEGF kit (R & D System DuoSet ELISA Development kit, cat. No DY493) according to the manufacturer's protocol.

The rate of VEGF protein production (PR_{VEGF}) by LLC cells was calculated using the formula (2) by numerical integration method.

$$PR_{VEGF}(\Delta t_i) = \frac{V \times \Delta C_{VEGF}}{\int_{t(i)}^{t(i+1)} N(t) \times dt} \text{ pg/h/}10^6 \text{ cells}, \quad (2)$$

where $PR_{VEGF}(\Delta t_i)$ — VEGF production rate (mean value) in the range time from $t(i)$ to $t(i+1)$; ΔC_{VEGF} — differences between VEGF concentration at time $t(i+1)$ and it concentration at time $t(i)$; V — the culture medium volume; $N(t)$ — number of viable cells ($X + Y$) at time point t in the range $\{t(i), t(i+1)\}$.

To analyze VEGF stability 3-day cell culture supernatants was collected and incubated during 7 days at 37 °C. Daily measurements of VEGF in supernatant aliquots as described above showed that changes of its level during 7 days was less than 5% of the initial one.

Mathematical modeling of growth kinetics of cancer cells. The phenomenological model of the growth kinetics of a proliferative heterogenic cancer cell population detailed in [5, 20] is used as the basic mathematical model. The main biological assumptions underlying of the model are briefly as follows (Fig. 1):

- The cancer cell population consists of proliferating, quiescent and dead cells;
- Under depletion of nutritional substrates the cells lose their ability to divide. In that case the cells transit from the stage of proliferation to the resting stage;
- The rate of cell transition from proliferation to quiescence depends upon the concentration of the main energy substances in cell incubation medium such as oxygen and glucose. Since oxygenation in experimental conditions was constant, function of cell transition from proliferative state to quiescence is considered to depend upon solely the concentration of glucose in cell incubation medium;
- Quiescent cells can be involved into proliferation (under action of mitogenic factors) or may die;

• Cells are growing in monolayer (two-dimensional growth), which provide for all cells equal availability to nutrient substances;

• Cells are not exposed to the exogenic cytotoxic (cytostatic) agents and mitogenic factors.

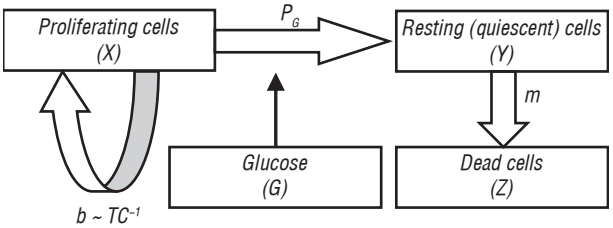

Fig. 1. Block-scheme of mathematical model for 2-D growth kinetics of cancer cells

In the framework of such assumptions the growth kinetics of cancer cell population can be described by the following system of differential equations (1):

$$\begin{cases} dX/dt = b \times X - P_G \times X \\ dY/dt = P_G \times X - m \times Y \\ dZ/dt = m \times Y \\ dG/dt = -(a_1 \times X + a_2 \times Y)/V \\ P_G = k_1 \times \exp(-k_2 \times G), \end{cases} \quad (1)$$

where ***X, Y, Z*** — the number of proliferating, resting and dead cells at point of time ***t (i)***, correspondingly; ***G*** — glucose concentration; ***b*** and ***m*** — proliferating and death rates, correspondingly; a_1 and a_2 — consumption rate of glucose by proliferating and resting cells, correspondingly; ***V*** — culture medium volume. It should be mentioned that proliferating rate (parameter ***b***) of cancer cells in the framework of exponential stage of monolayer growth is inversely proportional to the duration of the mitotic cycle (T_C) with the constant of proportionality close to 1.0. The function of cell transition from proliferating to resting state ***(P_G)*** is assumed to depend on glucose concentrations. Parameter k_1 equals to the maximum value of transition rate. Parameter k_2 reflects the glucose sensitivity of transition rate.

In order to estimate the model parameters, reflecting kinetic characteristics of cell growth and proliferative heterogeneity, a non-linear regression program, which is based on the SLEXIPLEX procedure, has been used. In the framework of the fitting algorithm for every step of the sliding in the parameter space the numerical solution of the system (1) has been calculated on the interval {0,t(N)} by Eiler method (where N is a number of observed data) [5].

The statistical analysis of the results was carried out using descriptive methods, *t*-test, correlation analysis and nonlinear regressive analysis.

RESULTS AND DISCUSSION

The results of the study of growth kinetics of LLC cells and dynamics of alteration of glucose level in incubation medium of the cells grown as unfed culture for 5 days are presented on Fig. 2. The study has shown that changes in the number of viable LLC cells possesses non-monotonous character — during first two-three days of growth the number of cells rose exponentially, with following decrease after the maximal level was achieved. At the same time the decrease of number of viable cells and increase of number of dead cells has been observed upon complete depletion of culture medium by glucose content; this result is in agreement with hypothesis on an important role of glucose for proliferative activity of LLC cells. Really, apart from the fact that glucose as well as oxygen is the main energetic substrate, it is also a basic plastic substrate — more than 80% of all phosphorus-containing cell metabolites are synthesized from glucose.

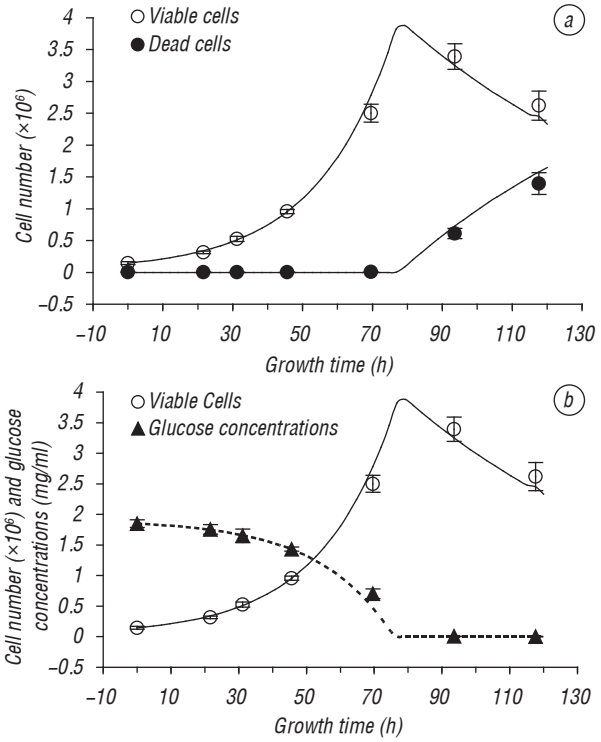

Fig. 2. The growth kinetics of LLC cells (*a*) and changes of glucose level (*b*) in incubation medium during cell growth in unfed culture. Symbols — experimental data; lines — model approximation obtained from the best fit of mathematical model (1) to experimental data

The fact that observed growth inhibition of LLC cells is resulted from the substrate depletion of culture medium (where glucose level may be considered at least as a marker of this depletion) rather than from cell density inhibition of cell proliferation is confirmed by the results of the investigations of growth kinetics of LLC cells growing in culture medium with nutrients redundancy ("fed culture"). As it seen in Fig.3 in the lack of nutrient deficiency LLC cells display exponential growth amounting to 20×10^6 cells per dish during 4 days of growth, that is about 2.5 times more than confluent monolayer number (coming to about 6.5×10^6). In "unfed culture" LLC cells show growth deceleration beginning from 48 h that is synchronized with a decrease of glucose concentration lower than physiological level (which is at close range to 0.9 mg/ml). It should be noted that during LLC growth as "fed culture" (as well as "unfed culture") overwhelming majority of cells grew on the plastic — number of viable detached cells counted in culture medium was less than 7% of the total viable cells per dish.

Obtained results completely correspond to peculiarities of malignant cells. Really it is well known that

deregulation of main cellular functions (proliferation, differentiation and cell motility) is an attribute of malignant cells [21]. Intracellular mechanisms of normal cells provide for both contact inhibition of cell motility and inhibition of cell proliferation due to differentiation or high cell density. In contrast to normal cell the malignant one is characterized by the absence of contact inhibition of cell motility (moreover may display contact-activated migration [22]) and the abnormalities of differentiation processes. Furthermore from the set of intracellular mechanisms resulting in high density inhibition of normal cell proliferation including cohesion/contact (substrate independent) mechanisms and substrate-dependent (resulted from the decrease in availability of cells to nutrient) mainly the latter is realized in cancer cells [23]. That's why cancer cells manifest exponential 3-D growth under the absence of substrate deficit.

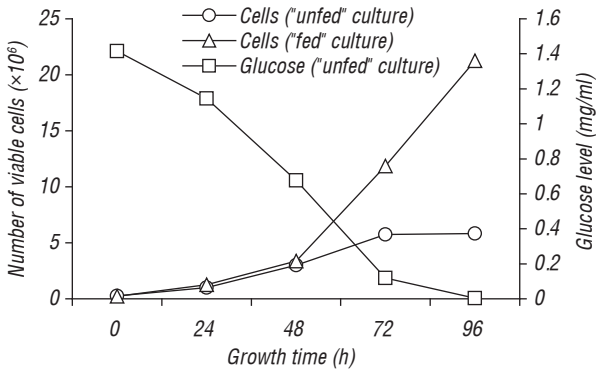

Fig. 3. Growth kinetics of LLC cells cultivated under deficit ("unfed" culture) or redundancy ("fed" culture) of nutrients

As one may see on Fig. 2, model (1) precisely describe growth kinetics of tumor cell population upon their growth without replacement of culture medium; which allowed to determine growth kinetic parameters (model parameters) of LLC cells and to predict the changes in proliferative heterogeneity. The rate of LLC cell division (which is determined by ratio ln (2)/b) is equal to 14.85 ± 1.5 h (Table 1). Glucose dependence of cell transition rate from proliferation to resting state possesses a pronounced two-phase character (Fig. 4, *a*). At a wide range of relatively high glucose concentrations (> 0.7 mg/ml) the transition rate is quite small. At concentrations lower than 0.7 mg/ml, the rate of transition swiftly increases (Fig. 4, *b*) resulting in sharp change in cellular composition (Fig. 5). It should be mentioned that $\boldsymbol{P_G}$ reflects a fraction of dividing cells switching to resting state. The real number of cells that pass to quiescent state for the day at glucose level ***G*** equals to the product of transition rate and number of dividing cells at the same glucose level. For instance when medium glucose concentration is about 0.7 mg/ml the transition rate is higher than 10^{-5} (day^{-1}) and number of quiescent cells appeared for the day varies from 20 to 5000 (taking into account the statistical errors of parameters $\mathbf{k_1}$ and $\mathbf{k_2}$). Glucose concentration about 1.0 mg/ml (at which transition rate is less than 10^{-6} day^{-1}) can be considered as the threshold value for single cell reduction of which stimulates the transition of cell from proliferating to resting state.

Table 1. Growth kinetic parameters of LLC cells growing as unfed culture

Growth kinetic parameters	Units	Value (M ± m)
b	Day^{-1}	1.12 ± 0.12
k_1	Day^{-1}	9.7 ± 4.2
k_2	$(mg/ml)^{-1}$	19.8 ± 8.7
a_1	$mg/day/10^6$ cells	4.1 ± 0.6
a_2	$mg/day/10^6$ cells	< 0.1 ± 0.06
m	day^{-1}	0.3 ± 0.05

At an interval from 70 to 90 h, practically all proliferating cells transit to a resting state. The rate of quiescent cell death is relatively low, and this is in part caused by too low level of glucose consumption compared to proliferating cells. These two facts are providing slow decrease of the pool of quiescent cells in the population, thus significantly increasing their chance to survive upon nutritional deficiency.

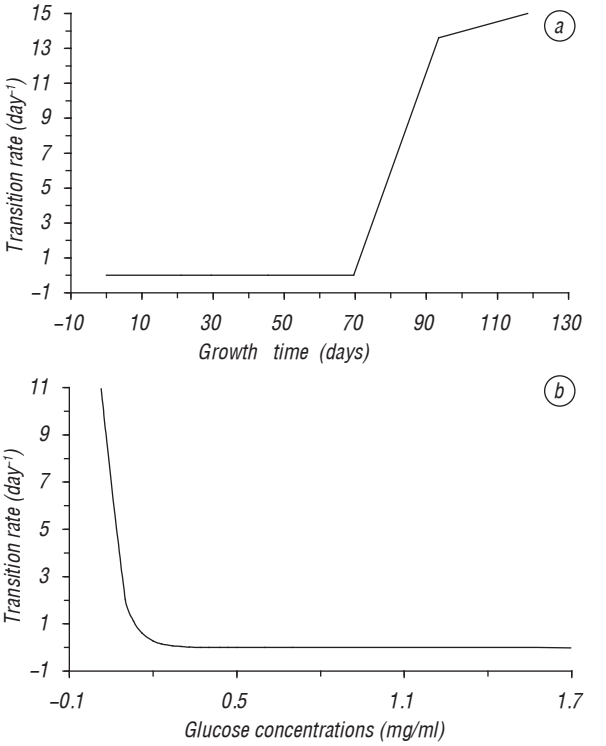

Fig. 4. LLC transition rate from proliferating to resting state as a function of growth time (*a*) and glucose level in incubation medium (*b*) predicted by the mathematical model (1)

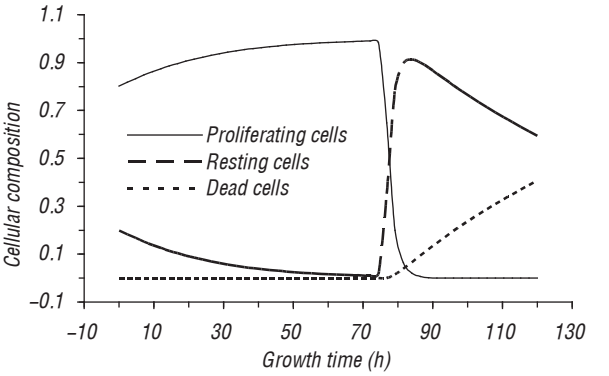

Fig. 5. The changes of cellular composition during LLC growth in unfed culture (model prediction)

It was shown that during LLC cells growth the level of VEGF in incubation medium is increasing monotonously (Fig. 6). Evaluation of VEGF production rate (*PRVEGF)* has shown monotonous decrease of this index (Fig. 7). Observed decrease of PR_{VEGF} could not be explained by VEGF degradation in incubation medium

(our results displayed the stability of VEGF molecule during investigations). Decrease of VEGF production rate, at least at first three days of LLC cells growth could nor be explained by the changes in cell number and cellular composition, because at this period elevation of the volume of LLC cell population is completely determined by the growth of its actively proliferating subpopulation at constant number of quiescent cells and the absence of cell death. Fall of VEGF production rate may result from either decrease in glycolysis intensity (due to glucose level reduction) or inhibition by VEGF itself (through allosteric mechanism). Interestingly that our results showed that VEGF production rate may depend on LLC cell seeding density. As it seen in Table 2 the PR_{VEGF} of LLC cells seeding at decreased density (by 33%) in 24 h was on 32% ($p < 0.05$) higher than that of cells seeding at greater density. Such inverse dependence is justified in the framework of "growth strategy" of cancer cells, because VEGF was shown to promote cancer cell survival and proliferative activity via paracrine and/or autocrine mechanisms [24, 25]. Meanwhile the mechanisms underlying this phenomenon are not totally clear.

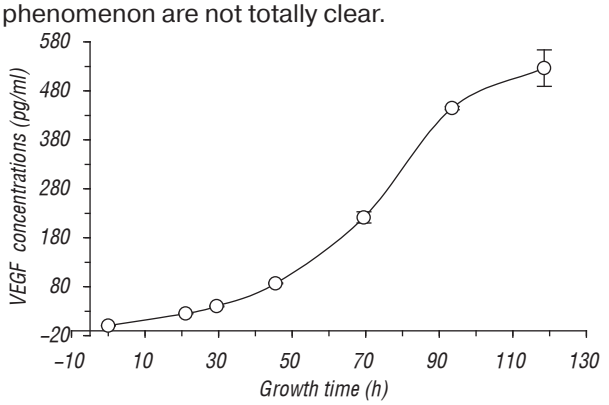

Fig. 6. The elevation of VEGF level in incubation medium during LLC growth in unfed culture. Symbols — experimental data, line — B-spline approximation

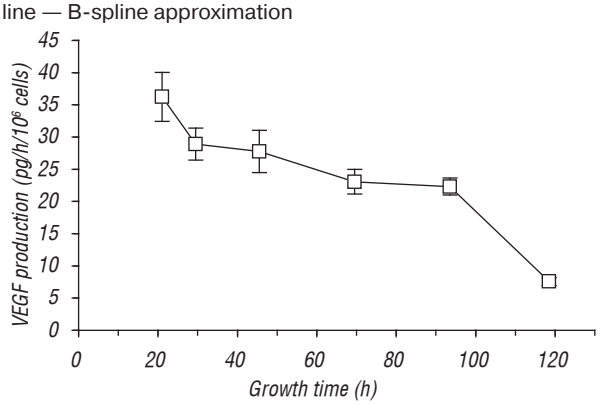

Fig. 7. Changes of VEGF production rate during LLC growth in unfed culture. Symbols — data calculated using formula (2); line — straight line approximation

Table 2. Influence of LLC cell density at the beginning of the growth on VEGF production rate

Number of viable cells (10^6)		VEGF concentrations after 24 h pg/ml	VEGF production rate pg/ (10^6 cells × h)
0 h	24 h		
0,110 ± 0,005	0.24 ± 0.005	37.4 ± 2.9	63.0 ± 3.0
0,166 ± 0,026	0,51 ± 0.052	54,3 ± 4.2	47,4 ± 7.3

It should be mentioned that growth of LLC cells in unfed culture is accompanied by changes in a wide spectrum of growth medium characteristics (biochemical and chemical composition, physical properties). Each of these factors can affect VEGF production rate of LLC cells. For instance it is well known that changes in pH level may alter VEGF production rate through modulation of VEGF expression [26–28]. However in a background of minor changes in growth medium occurred during first 24 h it is difficult to explain such significant and quite rapid changes in production rate of LLC cells by modulation of gene expression. Most likely it should be mediated through posttranscriptional events. After three days of growth a significant decrease of actively proliferating cells upon slight changes of viable cell number contributes for more progressive decrease of $PR_{VEGF.}$

So, the carried out investigations showed that elaborated mathematical model provided an adequate qualitative and quantitative description of the behavior of cancer cells growing as "unfed culture" and gave the possibility to predict the influence of glucose level on proliferative heterogeneity of cancer cell populations. Analysis of LLC growth kinetics by mathematical model has shown that LLC cells possess high proliferative activity at wide concentration range of glucose in culture medium. Significant inhibition of proliferation is observed at glucose content lower than 0.3 mg/ml, i. e. at concentrations 3-fold lower than physiologic one. At mentioned low concentrations of glucose the rate of cell death is quite small, providing high level of LLC cell viability upon nutritional deficiency. Such dependence between LLC cell survival (and proliferative heterogeneity) and glucose level may indicate a tolerance of LLC to metabolic stress, originated in a background of tumor angiogenic inhibition. This prediction is confirmed by the results of the investigations of anticancer activity of aconitine-containing agent BC1 with antiangiogenic mechanism of action [29] that showed the low efficacy of this agent against LLC [30].

It should be noted that the study of growth kinetics and proliferative heterogeneity of cancer cells growing as unfed culture could be considered as experimental model for development of the methods for estimation of sensitivity of tumor cell to antiangiogenic cancer therapy.

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АНАЛИЗ КИНЕТИКИ РОСТА И ПРОЛИФЕРАТИВНОЙ ГЕТЕРОГЕННОСТИ КЛЕТОК КАРЦИНОМЫ ЛЕГКОГО ЛЬЮИС, РАСТУЩИХ БЕЗ СМЕНЫ ПИТАТЕЛЬНОЙ СРЕДЫ

Цель: **провести анализ кинетики роста и пролиферативной гетерогенности клеток карциномы легкого Льюис (LLC) при их росте в монослое на протяжении 5 сут без замены культуральной среды (unfed culture).** ***Методы:*** **иммуноферментный метод определения уровня продукции VEGF опухолевыми клетками; глюкозооксидазный метод определения уровня глюкозы в среде инкубации; математическое моделирование.** ***Результаты:*** **предложенная математическая модель хорошо описывает экспериментальные данные, что позволяет определить кинетические параметры роста клеток LLC и предсказать изменения количества пролиферирующих и покоящихся клеток (пролиферативная гетерогенность) в процессе их роста. Кинетика роста клеток LLC носит немонотонный характер — в течение первых 3 сут их роста количество клеток экспоненциально увеличивается и далее, по достижении максимальной плотности, снижается. В то же время уменьшение количества живых клеток/увеличение количества мертвых клеток тесно связано с истощением глюкозы в среде инкубации. Зависимость скорости перехода клеток из состояния пролиферации в состояние покоя от содержания глюкозы в среде инкубации имеет выраженный двухфазный характер. В широком диапазоне относительно высоких концентраций глюкозы (> 1,0 мг/мл) скорость перехода близка к нулю. При концентрациях < 0,7 мг/мл скорость перехода стремительно возрастает, что приводит к резким изменениям клеточного состава клеточной популяции. В интервале от 70 до 90 ч практически все пролиферирующие клетки переходят в состояние покоя. Скорость гибели покоящихся клеток относительно низкая, что, в частности, связано с низким уровнем потребления глюкозы этими клетками по сравнению с таковым пролиферирующими клетками. Установлено, что в процессе роста скорость продукции VEGF клетками LLC монотонно снижается, несмотря на то, что в среде инкубации его уровень монотонно нарастает.** ***Выводы:*** **низкая зависимость скорости перехода клеток LLC из состояния пролиферации в состояние покоя от уровня глюкозы (> 0,7 мг/мл) в среде инкубации, а также низкий уровень их гибели обусловливает медленное снижение пула покоящихся клеток в популяции, что значительно повышает их шансы выжить в условиях дефицита питательных субстратов.**

Ключевые слова: **карцинома легкого Льюис, кинетика роста, глюкоза, пролиферативная гетерогенность, математическое моделирование.**