CISPLATIN–RESISTANT LEWIS LUNG CARCINOMA CELLS POSSESS INCREASED LEVEL OF VEGF SECRETION

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Aim: to reveal the differences in the production of vascular endothelial growth factor (VEGF) by Lewis lung carcinoma (LLC) and its cisplatin-resistant variant (LLC/R9) and to study the biological correlates between these differences and metastasis. Results: in vitro investigations showed that in the absence of cisplatin treatment cisplatin-resistant LLC/R9 cells produced increased level of VEGF in comparison with parental cells. The treatment of LLC/R9 cells with cisplatin in relatively low concentrations resulted in the decreased level of VEGF production. Meanwhile the same concentrations of cisplatin enhanced VEGF production by LLC cells. In vivo the dynamics of serum VEGF level in LLC-bearing mice was found to differ from that in mice with LLC/R9 by lower level of VEGF and by the existence of an essential lag time in the production of this factor. Unlike LLC, the dynamics of serum VEGF level in LLC/R9-bearing mice was characterized by absence of time delay and an increased level of VEGF. It has been shown that such differences in circulating VEGF are accompanied by the distinction in metastatic lung injury. The volume of lung metastases in mice with LLC/R9 was considerably higher than that in the animals with LLC, although the maximum number of lung metastases in animals with resistant tumor was by 22% less than in mice with parental one. Conclusion: Revealed changes in the secretion of VEGF give ground to suggest that intracellular mechanisms, providing increased production of VEGF by Lewis lung carcinoma cells in response to cisplatin action, are part of the adaptive reactions of tumor cell and underlie the formation of drug resistance of malignant tumors.

Key words: VEGF, drug resistance, metastasis, cisplatin, Lewis lung carcinoma.

One of the main and distinctive features of malignant tumors that considerably restrict the efficacy of cancer therapy is their ability to progress towards the appearance of drug resistance, invasion and metastatic spread of cancer cells. In fact, tumor metastasis remains the major cause of death for cancer patients. At the same time tumor drug resistance results in low efficacy of anticancer agents and continues to be a major and largely unsolved problem of cancer therapy. Despite the availability of approximately 60 different agents for the systemic therapy of cancer, refractoriness to treatment develops in the majority of cases.

Targeting of tumor angiogenesis represents a new strategy for the development of anticancer therapies that prolong or stabilize the progression of tumors [1]. The hypothesis that the process of the formation of new blood vessels from preexisting vasculature (tumor angiogenesis) can be considered as a target for anticancer therapy, was firstly proposed in 1971 [2] and is currently under intense experimental and clinical investigations [3–7]. The progress of antiangiogenic therapy was supposed to be connected with its low toxicity against normal tissue and its efficacy against drug resistant tumors [8]. In 1991, the data on the cytotoxicity of antiangiogenic agents against drug-resistant solid tumors have been reported [9].

In spite of the fact that over 75 antiangiogenic agents are presently in clinical trials, none is currently approved for cancer therapy. Analysis of the results obtained in experimental investigations and clinical trials showed that there are several obstacles for the use of antiangiogenic drugs limiting the efficacy of antiangiogenic therapy [10], i.e. the establishment of the appropriate doses and scheduling of antiangiogenic drugs, revelation of biological correlates and determination of the best ways to combine these treatments with chemotherapy. Moreover, the regimens of antiangiogenic therapy significantly depend on the angiogenic profiling that is predominantly produced by tumor cells [1]. Indeed angiogenic profiles may be different for tumors of different origin and different sensitivities against chemotherapeutic drugs [11]. As far as metastatic process is accompanied by substantial changes of cellular composition of malignant tumors [12, 13] changes in angiogenic profile should be expected during growth and metastasis of malignant tumors.

To overcome the above-mentioned obstacles, further preclinical research is required in order to reveal reliable biological correlates for determining the optimal antiangiogenic treatment. It is well recognized now that among a number of proangiogenic factors produced by cancer cells, vascular endothelial growth factor (VEGF) plays a critical role in tumor-associated angiogenesis promoting tumor growth, invasion and metastasis [14].

That's why our work is aimed on revealment of the differences in the production of VEGF between Lewis lung carcinoma (LLC) and its cisplatin–resistant variant and to study the biological correlates between these differences and metastasis.

MATERIALS AND METHODS

Experimental animals and tumor strains. The study was carried out on 2–2.5 months old C57Bl/6 mice.
mice weighing 20–23 g obtained from the vivarium of R.E. Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology of NAS of Ukraine (IEPOR) (Kyiv, Ukraine). All investigations with animals were performed according to the rules of Ethic Committee.

Lewis lung carcinoma (LLC) cells used as tumor cells sensitive to cisplatin (their IC₅₀ for cisplatin was 0.008 ± 0.0027 mg/ml) were obtained from the National Bank of Cell Lines and Tumor Strains (IEPOR, Kyiv, Ukraine). Cisplatin-resistant LLC/R₉ cells (IC₅₀ was 0.013 ± 0.002 mg/ml) were kindly gifted to us by prof. V.F. Chekhun from the Department of Anticancer Therapy of IEPOR (Kyiv, Ukraine).

**In vivo experiments.** LLC and LLC/R₉ cells (10⁶) were transplanted by intramuscular injection to 14 and 27 animals, respectively. Animals were examined and blood serum samples were taken at the next time points: LLC-bearing mice – at days 13, 17, 21, 24 and 28 after tumor cell inoculation, LLC/R₉-bearing mice – at days 10, 15, 20, 24 and 29 after tumor cell inoculation (3–7 animals per each time point).

VEGF levels in blood serum were determined by immunoassay as described in [15].

The growth kinetics of primary tumors as well as the volume and total number of lung metastases were evaluated by routine methods. The number of lung metastases (as a portion of their total number per mouse) was also analyzed in vascular phase (metastasis volume > 1 mm³).

**In vitro study.** The level of VEGF production was determined in R and P–cells variants, obtained from cisplatin-resistant LLC/R₉ and parental LLC correspondingly by routine trypsin disaggregation of primary tumor tissues.

Both R and P–cell variants were plated into 96–well plates (Nunclon, Denmark) at the density 2 x 10⁴ cells/well and cultured in RPMI 1640 (Sigma, USA) (in a volume 0.2 ml/well) supplemented with 2 mM glutamine, 10% FBS and 40 µg/ml gentamicine at 37 °C in humidified atmosphere containing 5% CO₂. Cytotoxicity of cisplatin in the range from 0.5 mg/ml to 0.0005 mg/ml was evaluated by the measurement of the number of viable cancer cells by MTT calorimetric assay [16]. After 24 h incubation, samples of culture medium were collected, the number of viable cells was determined in units of extinction (E₅₄₀), and VEGF level was measured by immunoassay [15]. Cell–free complete culture medium and negative control cell culture (nontreated) were used as controls.

The level of VEGF production per cell was calculated by formula VEGFₖₑₜₙ = VEGFₙₐₜ/E₅₄₀, where VEGFₙₐₜ is the amount of VEGF per cell; VEGFₙₐₜ – the total VEGF level in samples of culture medium (ng/ml), and E₅₄₀ reflects the number of viable cells per well; the index was expressed in relative units.

All investigations were carried out in triplicate.

**The statistical analysis** of the results was performed using descriptive methods, Student’s t–test, correlation analysis and nonlinear regressive analysis.

**RESULTS AND DISCUSSION**

Scanty investigations have shown the increase of the level of proangiogenic factors secretion by tumor cells resistant to antiangiogenic therapy [17]. The results of our investigations revealed that LLC cells resistant to cisplatin are also characterized by increased level of VEGF production.

*In vitro* it was found that the level of VEGF production by R–cells derived from LLC/R₉ was 2.6 times higher (p < 0.001) than that by P–cells (Fig. 1).

![Fig. 1. The level of in vitro VEGF production by P– (1) and R–cells (2)](image)

Fig. 1. The level of in vitro VEGF production by P– (1) and R–cells (2)

*In vivo* study of serum VEGF level in LLC/R₉–bearing mice confirmed the results obtained *in vitro*. The dynamics of the serum VEGF level in mice bearing cisplatin–resistant tumor significantly differs from that in mice with the parental LLC, and is characterized by a higher level of VEGF production, at least at the initial phase of the primary tumor growth (Fig. 2).

![Fig. 2. Changes of serum VEGF level in LLC and LLC/R₉–bearing mice](image)

Fig. 2. Changes of serum VEGF level in LLC and LLC/R₉–bearing mice

To compare VEGF levels in cisplatin–resistant and sensitive tumors, we analyzed the dynamic changes of proangiogenic factor using Weibull’s function:

\[
\text{VEGF} = \text{VEGF}_0 \times \exp(\alpha \times (t - \tau_{lag}))
\]  

(1)

In the framework of Weibull’s model the dynamic changes of the serum VEGF level are assessed by three main parameters. The parameter \(\alpha\) reflects the level of VEGF secretion per cell. \(\beta\) considers the influence of

**Table.** Parameters of the model for the dynamic changes of VEGF level in blood serum of LLC and LLC/R₉–bearing mice during tumor growth

<table>
<thead>
<tr>
<th>Tumor type</th>
<th>(\alpha) (days⁻¹)</th>
<th>(\beta)</th>
<th>(\tau_{lag}) (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LLC</td>
<td>1.66 ± 0.15</td>
<td>0.2 ± 0.036</td>
<td>17.0 ± 0.3</td>
</tr>
<tr>
<td>LLC/R₉</td>
<td>2.1 ± 0.07*</td>
<td>0.08 ± 0.01*</td>
<td>0.0 ± 0.0*</td>
</tr>
</tbody>
</table>

* p < 0.01.
the cellular composition of the primary tumor on VEGF production by single cells. $t_{lag}$ is a delay in the serum VEGF level produced by entire tumor. $VEGF_0$ is the level of VEGF in the blood serum of intact animals, and for C57Bl/6 mice it was equal to $11.3 \pm 1.5$ ng/ml.

The parameters of the model for cisplatin–resistant and parental tumors were determined from the best fit of mathematical model (1) to experimental data using nonlinear regression analysis.

Weibul’s mathematical model adequately describes VEGF dynamic curves for both parental and resistant tumors (Fig. 2), thus indicating that the changes of serum level of VEGF during LLC and LLC/R9 growth result from the same intracellular, intercellular and intra–tumor processes.

Comparison of the model parameters has showed that the changes in the level of circulating VEGF during LLC/R9 growth significantly differed from that for LLC. This is testified by the presence of significant differences in all parameters of the mathematical model. At the cellular level these differences are due to the ability of the cisplatin–resistant cells to produce an increased VEGF level, which is supported by differences in the parameter $\alpha$. This result is in good agreement with in vitro data obtained for R–cells.

An interesting feature of LLC (which makes it different from cisplatin–resistant variant) is the existence of an essential lag time (equal to 17 days) in the production of VEGF, not detected in LLC/R9–bearing mice. One cannot explain such delay by low level of VEGF secretion by P–cells (compared to their resistant counterpart) due to the lack of significant correlation between the dynamic changes in VEGF levels and the kinetics of LLC primary tumor growth (Fig. 3, a). Meanwhile, such a delay of VEGF level may result from the cellular heterogeneity of LLC in the framework of which only part of tumor cells actively produces VEGF ($VEGF^+–$subpopulation). Apparently, at the beginning of tumor growth the cells of this subpopulation constitute the minority of LLC cellular composition. Increase of VEGF level in LLC–bearing mice at day 17th may be associated with a progressive increase of VEGF$^+–$subpopulation. The production of VEGF provides the cells of this subpopulation with growth advantages that are necessary for dominating in heterogeneous tumor. The results of the latest investigations, which showed that VEGF in addition to its key role in tumor angiogenesis, is also one of the important factors in the survival of cancer cells support the mentioned above point [18–20]. In particular, using the cell line of human ovarian carcinoma (which expresses high and stable level of VEGF–164) it was shown that the expression of VEGF markedly increases the survival of cancer cells both in vitro and in vivo and conditions on their resistance to apoptosis induced by cisplatin [18]. In [19] VEGF–C secreted by human leukemia cells was shown to stimulate cell proliferation and protect cancer cells from apoptosis induced by cytotoxic agents through interaction of this proangiogenic factor with cancer cell receptor VEGFR–3 (FLT–4).

The importance of VEGF production for the survival of tumor cells was supported by our findings obtained in vitro. Thus, we showed that effect of cisplatin in concentrations less than IC$50$ on P–cells resulted in increased VEGF production (Fig. 4), which apparently can be considered as a part of the adaptive response of the cell toward the cytostatic agent. One may suggest that at least some part of VEGF subpopulation consists of highly metastatic cells. Re–
ally it is known that in the framework of metastatic cell dominance phenomenon [12, 21], the appearance of distant metastases is preceded by the dominance of highly metastatic cells. Our previous investigations showed that exactly the 17th day is a particular time point when LLC predominantly consists of highly metastatic cells [13]. So, the increase in serum VEGF level may be associated with active production of VEGF by mentioned cells. The ability to produce the proangiogenic factors by metastatic cells enhances their growth advantages that are already peculiar to these cells and are known to be necessary for them in order to dominate in heterogeneous tumor [12, 21–23].

Unlike LLC, the changes in serum VEGF level in LLC/R9-bearing mice during tumor growth are characterized by the absence of time delay (tlag=0) and an increased level of circulating VEGF even at the early stages of tumor growth (Table, Fig. 2). If our mentioned considerations are correct, then cisplatin-resistant tumor at the initial stage of growth contains higher volume of VEGF+-subpopulation than LLC. This conclusion is supported by high and significant correlation (r = 0.74, p < 0.01) between the dynamic changes of serum VEGF level and LLC/R9 growth kinetics (Fig. 5, a) (in case of LLC such correlation was absent), and by the shift in LLC/R9 metastasis curve to the left (Fig. 5, b). By day 25, the number of lung metastases was significantly higher (p < 0.05) in LLC/R9-bearing animals than in mice with LLC.

Nevertheless, it should be noted that in both resistant and parental tumors only part of the cells of VEGF+-subpopulation possesses high metastatic activity. This is evidenced, by the continuous increase of serum VEGF level, and by the progressive decrease of the number of metastatically active cells at the late stages of tumor growth [13].

Differences in the serum VEGF level between LLC- and LLC/R9-bearing mice are accompanied by the distinction in metastatic lung injury. There is a significant correlation between the blood serum VEGF level and the number of lung metastases for LLC and LLC/R9 tumors (r = 0.77, r = 0.64, p < 0.01 respectively). However, LLC/R9-bearing mice despite significantly (p < 0.05) higher maximal serum VEGF level (177.6 ± 5.1 ng/ml against 162.3 ± 4.7 ng/ml in LLC), have significantly lower (p < 0.05) maximal number of lung metastases (20.1 ± 1.3) than mice with LLC (25.1 ± 1.9).

The possible explanation of that fact is that the serum VEGF level plays a critical role in the progressive metastatic growth [14, 24–26], while the number of metastases is mainly determined by the metastatic potential of primary tumor. This explanation is supported by a significant correlation observed between the level of circulating VEGF in mice with LLC and the number of metastases in vascular phase of their growth (r = 0.67, p < 0.01) (Fig. 3, c). In case of cisplatin-resistant tumor, such correlation is absent due to redundant serum VEGF level (which is higher than it is necessary...
for the induction of metastases (vascularization) during LLC/R9 growth. However, much higher VEGF level in mice with LLC/R9 accounts for a significantly higher (p < 0.01) level of lung metastatic injury than that in mice with LLC. The maximal volume of lung metastases in animals with LLC/R9 was 20.9 ± 2.9 mm³ (Fig. 3, d), while in mice with LLC – 5.1 ± 0.5 mm³ (Fig. 3, d).

It should be noted that though the volume of lung metastases in mice with LLC/R9 was significantly higher (p < 0.05) than that in animals with LLC, there was no significant correlation between the serum VEGF level and the total lung metastasis volume at all stages of tumor growth due to its negligible value (< 1% of the primary tumor volume).

In conclusion, we showed that cisplatin resistant LLC cells produce higher level of VEGF (in comparison with parental cells) in the absence of treatment. The in vitro treatment with cisplatin in relatively low concentrations resulted in the decrease of VEGF production by LLC/R9 cells but enhanced VEGF production by parental LLC cells. We suppose that intracellular mechanisms responsible for increased production of VEGF by LLC cells in response to cisplatin action, represent the adaptive reactions of tumor cell and underlie the formation of their drug resistance.

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ПОВЫШЕНИЕ УРОВНЯ ПРОДУКЦИИ VEGF РЕЗИСТЕНТОЙ К ДЕЙСТВИЮ ЦИСПЛАТИНА КАРЦИНОМЫ ЛЕГКИХ ЛЬЮИС

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Цель: данная работа посвящена сравнительному анализу уровня продукции фактора роста эндотелиальных клеток (VEGF) клетками карциномы легких Льюис (LLC) и ее цисплатин-резистентного варианта (LLC/R9) и выявлению связи между продукцией VEGF и метастазированием. Результаты: в опытах in vitro показано, что клетки LLC/R9 не подвергавшиеся воздействию цисплатина, характеризовались повышенным уровнем продукции VEGF по сравнению с клетками LLC. Под воздействием цисплатина в низких концентрациях наблюдалось снижение продукции VEGF клетками LLC/R9 и повышение продукции этого фактора клетками LLC. Исследования in vivo показали, что динамика изменения уровня VEGF в сыворотке крови мышей с LLC, в отличие от таковой у мышей с LLC/R9, характеризуется пониженным уровнем VEGF, а также наличием 17-суточного латентного периода в продукции этого фактора. Наряду с указанными различиями, исследуемые группы различались по степени метастатических поражений легких, объем которых у мышей с LLC/R9 был значительно выше, чем у мышей с LLC, хотя максимальное количество метастазов у животных с резистентной опухолью было на 22% меньше, чем у мышей с исходным штаммом. Выводы: обнаруженные изменения в продукции VEGF дают основание предполагать, что внутриклеточные механизмы, обеспечивающие повышенную секрецию этого фактора клетками карциномы Льюис в ответ на действие цисплатина, могут являться частью адаптивных реакций опухолевой клетки и лежать в основе формирования лекарственной резистентности злокачественных опухолей.

Ключевые слова: фактор роста эндотелиальных клеток, резистентность, метастазирование, цисплатин, карцинома легких Льюис.