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SPONTANEOUS APOPTOSIS AND PROLIFERATIVE ACTIVITY *IN VITRO* OF LEUKEMIC CELLS FROM CHILDREN WITH ALL: RELATIONSHIP WITH *IN VITRO* SUSCEPTIBILITY TO ANTICANCER DRUGS

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СПОНТАННЫЙ АПОПТОЗ И ПРОЛИФЕРАТИВНАЯ АКТИВНОСТЬ *IN VITRO* ЛЕЙКЕМИЧЕСКИХ КЛЕТОК ДЕТЕЙ С ОЛЛ: СВЯЗЬ С ЧУВСТВИТЕЛЬНОСТЬЮ К ПРОТИВОРАКОВЫМ ПРЕПАРАТАМ

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In the work, the relation between apoptosis/proliferation activity indexes and chemosensitivity of tumor cells *in vitro* in pediatric patients with ALL has been investigated. We have shown that the decreased ability of tumor cells to undergo spontaneous apoptosis is accompanied by the increase of resistance to cytarabine and L-asparaginase. No relation between expression levels of CD95, CD95L and bcl-2 proteins and cell sensitivity to anticancer drugs was found. The reverse corellation was demonstrated between bcl-2 expression and spontaneous apoptosis level, as well as between LC50 values for L-asparaginase, or vepeside and the number of cells at $S+G_2M$ phases. The decrease of S/G_2M relation was accompanied by increase of tumor cells resistance against rubomicine, doxorubicine and cytarabine.
***Key Words*: drug sensitivity, cell cycle, spontaneous apoptosis, CD95, CD95L antigens, bcl-2 protein.**

В статье представлены данные о связи между показателями апоптоза и пролиферативной активности *in vitro* с чувствительностью к лекарственным препаратам клеток при остром лимфобластном лейкозе (ОЛЛ) у детей. Показано, что сниженная способность опухолевых клеток подвергаться спонтанному апоптозу сопровождалась повышением резистентности к цитозару и L-аспарагиназе. Не было выявлено связи экспрессии антигенов CD95, CD95L и белка bcl-2 с чувствительностью лейкемических клеток к противоопухолевым препаратам, но было установлено ингибирующее влияние bcl-2 на уровень спонтанного апоптоза. Отмечалась обратная корреляционная зависимость между LC50 L-аспарагиназы, LC50 вепезида и количеством пролиферирующих клеток, находящихся в $S+G_2M$ фазах клеточного цикла. Уменьшение соотношения S/G_2M сопровождалось повышением резистентности опухолевых клеток при ОЛЛ к рубомицину, доксорубицину и циторабину.
***Ключевые слова*: лекарственная чувствительность, клеточный цикл, спонтанный апоптоз, антигены CD95, CD95L, белок bcl-2.**

Polychemotherapy is routinely used for treatment of oncohematologic diseases. Despite certain achievements in therapy of acute lymphoblastic leukemia (ALL) in pediatric patients, the problem of the development of the antitumor drug resistance remains unresolved. The mechanisms of multiple drug resistance (MDR) development may depend on unability of cells to initiate apoptosis programms (decreased intracellular accumulation of cytotoxic agent, increase of DNA reparation rate etc) or by altered apoptosis programms caused by mutations of *p53* gene, hyperexpression of bcl–2 protein and inhibitors of apoptosis (IAPs), etc [1, 2]. Also, the decreased chemosensitivity of tumor cells may resulted from their extremely high proliferative activity — so called regrowth resistance [3]. So, the processes of cell apoptosis and proliferation play the crucial role in the development of anticancer drug resistance.

The work is aimed on the study of the relationship between apoptosis indexes, proliferative activity and sensitivity to anticancer preparations in children with ALL.

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Abbreviations used: ALL — acute lymphoblastic leukemia.

We have studied 64 ALL patients of 1 to 17 years old cured according to ALL–BFM–90M protocol in Republican Scientific Practical Center of Pediatric Oncology and Hematology (Mynsk, Belorussia) from 1999 to 2002. All studies were performed at the day of diagnosis.

Leukemic cells were isolated from the bone marrow of patients using the Histopaque density gradient (d = 1077). The sensitivity of tumor cells to drugs (dexamethasone (0.0002–6 μg/ml), prednisolone (0.008–250 μg/ml), vincristine (0.05–50 μg/ml), L–asparaginase (0.003–10 μg/ml), rubomycine (0.008–8 μg/ml), doxorubicine (0.008–8 μg/ml), cytarabine (0.01–10 μg/ml), vepeside (0.005–50 μg/ml)) was evaluated by the MTT test [4] using 3–(4,5–dimethylthiazol–2–yl)–2,5–diphenyltetrazolium bromide and expressed as concentration of the agent causing the death of 50% of cells (LC50).

The level of spontaneous apoptosis was evaluated in leukemic cells cultured in RPMI–1640 containing 15% FCS, L–glutamine, for 20 h as follows: 1) by fluorescent microscopy and staining with acridine orange and ethidium bromide (AO + EB); 2) for detection of cells with hypodiploid DNA content, the cells were stained with propidium iodide (PI); 3) the alteration of mitochondrial transmembrane potential (MMP) was tested us–

ing lipophilic cationic JC–1 probe Molecular Probes, the Netherlands); 4) the detection of DNA breaks was performed using APO–BRDU Kit (Becton Dickinson Pharmingen, USA). For the study, fluorescent microscope LUMAM РПО–11 (LOMO, Russia) and cytofluorimeter FACScan (Becton Dickinson, USA) were used.

To study cell cycle alterations, fixed leukemic cells were treated with RNAase, stained with PI solution [6], and analized using FACScan cytofluorimeter and CellFit program.

For the study of expression of surface (CD95, CD95L) and intracellular (bcl–2) molecules, monoclonal antibodies DX2, NOK–1, bcl–2/100 (B&D, USA) and FACScan cytofluorimeter were used.

For analysis of correlation, Spearmen's test was used. Statistical analysis of the data was performed using Statistica 5.0 program.

Corellation analysis has demonstrated that LC50 for cytarabine and L–asparaginase is reversely related with spontaneous apoptosis level: R = –0,52 (n = 21, p = 0.02 by means of MMP for cytarabine, R = –0,27 (n = 64, p = 0.03) by means of PI for subG1 peak for L–asparaginase. At the same time corellation index between LC50 of vincristine and apoptosis level detected by means of DNA breaks was R = 0.3 (n = 41, p = 0.04). Similar results were reported by Osipova et al [9] but for other agents. Taking to account that we did not receive significant corellation between LC50 of all studied preparations and spontaneous apoptosis level, one may suppose that mechanisms of initiation of spontaneous and drug–induced apoptosis are different.

Table. Relation between tumor cell sensitivity *in vitro* to chemopreparations and their distribution by cell cycle phases

Anticancer preparations	n	R and p values			
		S	G_2M	$S+G_2M$	S/G_2M
Dexametasone	48	0.15	–0.12	0.004	0.1
		0.3	0.4	0.9	0.4
Prednisolone	53	0.1	0.1	0.1	–0.02
		0.5	0.4	0.4	0.9
Vincristine	47	0.1	–0.2	–0.1	0.1
		0.4	0.2	0.5	0.4
L–asparaginase	50	–0.3	–0.2	–0.28	0.04
		0.5	0.3	0.05	0.8
Vepeside	27	–0.2	0.2	–0.29	–0.3
		0.2	0.4	0.06	0.1
Rubomycine	25	–0.18	0.5	–0.1	–0.5
		0.4	0.009	0.6	0.009
Doxorubicine	26	–0.3	0.38	–0.24	–0.48
		0.1	0.05	0.2	0.01
Cytarabine	27	–0.27	0.38	–0.1	–0.38
		0.17	0.05	0.7	0.05

Next, we have analyzed corellation of expression of apoptosis–mediating molecules CD95 и CD95L and intracellular antiapoptotic protein bcl–2 with the drug sensitivity of tumor cells *in vitro*. However, no statistically significant relations were revealed as well as no significant influence of CD95 and CD95L expression on the value of spontaneous apoptosis.

At the same time, it has been noticed that bcl–2 expression is reversely related to spontaneous apoptosis level *in vitro* (Figure). Corellation coefficient between the number of apoptotic cells (by means of morphologic analysis) and the level of bcl–2 expression was –0.48 (n = 20, p = 0.03). If MMP test was applied, R was –0.49 (n = 13, p = 0.08). As it was reported for AML cases [10], the cells with high bcl–2 expression are characterized by decreased spontaneous apoptosis levels, but not *vise versa*; i.e. the insrease of bcl–2 expression is not the only one cause of the decrease of spontaneous apoptosis in leukemic cells.

Taking to account that the majority of chemopreparations are directed against proliferating cells, the decreased proliferation rate of tumor cells may be among the causes of their drug–resistance. We observed the weak reverse corellation relation between LC50 of L–asparaginase and vepeside and the number of proliferating cells (the cells at $S+G_2M$ phases) – R = –0.28 (n = 50, p = 0.05), and R = –0,29 (n = 27, p = 0.06) respectively. Similar results were reported by other authors [11] for cytarabine, L–asparaginase, mercaptopurine, vincristine, thioguanine.

The data on relation between LC50 values and cell distribution by cell cycle phases are shown in a Figure. Despite the fact that no significant relations between LC50 and number of cells in S and $S+G_2M$ phases (except for L–asparaginase and vepeside), such corellation has negative value. From other hand, we have found out that the content of cells in G_2M–phase strictly corellates with their sensitivity to rubomycine, doxorubicine, cytarabine. The highest corellation values were between drug sensitivity and S/G_2M index; this relation is reverse ($p < 0.05$): the sensitivity of cells to rubomycine, doxorubicine, cytarabine is strictly proportional to content of cells in S–phase and reversely — to their content in G_2M–phase. One should note that no relation between drug sensitivity and cell phase distribution was found for prednisolone and dexamethasone

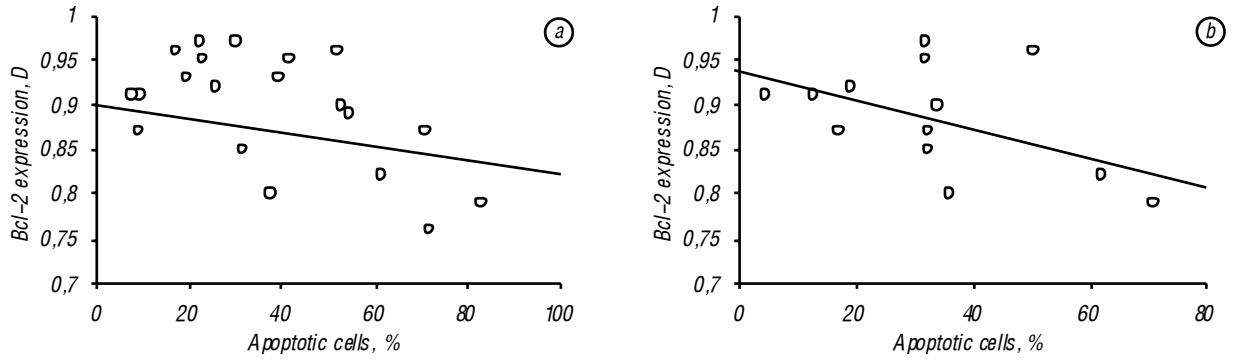

Figure. Corellation between the level of spontaneous apoptosis and expression of bcl–2 protein. The number of apoptotic cells was determined by morphological analysis of nuclei (*a*) or using MMP analysis (*b*)

that have no phase–specificity as well as for vincristine that is cytotoxic in mitosis.

Analysis of the relation between the level of spontaneous apoptosis level and distribution of cells by cell cycle phases has shown the reverse corellation between the number of cells in G_2M–phase and the number of cells with condensed chromatin (morphological analysis), where $R = -0.26$ ($n = 53$, $p = 0.06$). So, the decrease of sensitivity upon increase of the number of cells in G_2M–phase may be explained by the suppressed ability of those cells to initiate apoptosis.

In conclusion, the decreased ability of tumor cells to undergo spontaneous apoptosis was accompanied by increase of the resistance against cytarabine and L–asparaginase. CD95, CD95L and bcl–2 expression didn't influence the chemosensitivity of leukemic cells *in vitro*, but the latest index was in reverse correllation with spontaneous apoptosis level. The decrease of S/G_2M ratio is causing the increase of tumor cells resistance against rubomycine, doxorubicine and cytarabine.

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