Morphologic Changes in the Tumor and Liver in Mice with Transplanted RLS_{40} Lymphosarcoma during Increase of Its Drug Resistance

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The progress of drug resistance of RLS_{40} resistant lymphosarcoma and specific features of toxic lesions in the liver in polychemotherapy were studied. After intramuscular injection of tumor cells, the mice received a course of polychemotherapy. The tumor material was then collected and transplanted to intact animals, after which polychemotherapy was carried out. A total of 4 passages of tumor cells and 4 polychemotherapy courses were carried out. The expression of *mdr1b*, *bcl-2,* and *p53* genes in tumor cells increased by 1.3, 2.3, and 1.6 times after 4 courses of polychemotherapy in comparison with intact tumor. Volume density of apoptoses in tumor tissue after 4 polychemotherapy courses decreased 1.7 times compared to that after single course. The increase in cytostatic load was associated with aggravation of destructive changes in the liver: the volume density of necroses in the liver increased 1.3 times after 4 passages of the tumor.

Key Words: *resistant lymphosarcoma; polychemotherapy; drug resistance; hepatotoxicity*

Progress in antitumor therapy of hematological malignancies attained in recent decades led to an increase in the incidence of complete remissions, prolongation of relapse-free survival of patients, and in some cases to cure. These results are due to the use of effective polychemotherapy (PCT) protocols combining antitumor drugs with different mechanisms of action and toxic profiles and to introduction of molecular biological methods for verification of the tumor clone, due to which it is possible to carry out target therapy [2].

However, some unsolved problems impede the full-value and maximally effective use of programmed PCT. The first of these problems is high toxicity of multiple PCT courses [8] and the other is the formation of multiple drug resistance (MDR), acquisition of cross-resistance to cytostatics with different mechanisms of action and intracellular targets by the tumor cells [3]. These two problems can augment each other and significantly reduce PCT efficiency.

The resistance of tumor cell to PCT can result from a variety of processes from reduced intracellular concentration of antitumor drug because of substance release into extracellular space by P-glycoprotein (P-gp; ATP-dependent transmembrane protein), *MDR1* gene product, to disorders in the apoptosis mechanisms in tumor cells (*p53* gene mutation or deficit, impairing its proapoptotic function) and/or hyperexpression of *bcl-2* gene, causing insensitivity of cells to proapoptotic stimuli [7].

Studies of pharmacokinetics of antitumor drug suggest that they like the majority of drugs undergo biotransformation in the liver with the formation of toxic metabolites, which can cause hepatocyte damage [6]. Liver involvement is one of the main factors, along with bone marrow and myocardial involvement limiting complete programmed PCT, because the de-

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velopment of PCT complications in these organs, combined with endogenous intoxication syndrome (developing in the majority of malignant tumors [1]), can lead to lethal outcomes during PCT.

We studied the morphology of the tumor and liver in mice with progressive MDR of transplanted RLS_{40} lymphosarcoma during repeated courses of PCT.

MATERIALS AND METHODS

The study was carried out on 10-14-week-old male CBA/LacSto (CBA) mice bred at Institute of Cytology and Genetics. Drug-resistant murine lymphosarcoma RLS_{40} was transplanted to animals. This tumor initially exhibits the MDR phenotype corresponding to the tumor status in patients after chemotherapy or to the status of a tumor initially resistant to standard PCT protocols used as the first line therapy in the majority of hematological malignancies [7].

The RLS_{40} cell suspension in saline (5×10^6 cell/ml) was intramuscularly transplanted (0.1 ml) into the right hip for the formation of a solid tumor. On day 7 of tumor growth, the animals were divided into 2 groups. Group 1 mice received a standard combination of cytostatics (CHOP protocol) and group 2 mice received no therapy (control). The drugs were dissolved in saline directly before use and injected in doses corresponding to $^1/_5$ LD_{50}: single injections of cyclophosphamide (50 mg/kg), doxorubicin (4 mg/kg), and vincristine (0.1 mg/kg) into the caudal vein and prednisolone (5 mg/kg) intraperitoneally for 5 days. Controls were intraperitoneally injected with saline. On day 10 after treatment (day 17 of tumor growth), the mice were sacrificed by cervical dislocation under ether narcosis and tumor tissue was isolated for preparation of tumor cell suspension. A portion of the suspension was used for re-transplantation (passages) to intact animals (5×10^6 cell/ml; 0.1 ml), another portion was used for preparation of primary cell culture and evaluation of gene expression. On day 7 after implantation of the tumor subjected to PCT, the animals received a course of PCT (CHOP); controls were left without treatment. A total of 4 passages of tumor cells were carried out in mice treated with cytostatics.

Material for histological and molecular biological studies was collected on day 7 after cytostatic treatment after one passage of the tumor and on day 10 after the rest passages of the tumor and PCT courses.

The nodes from the hip and liver of experimental animals were fixed in 10% neutral formalin for histological study. The material was then dehydrated in ascending alcohols, clarified in xylene, and embedded in paraffin. Paraffin sections (up to 5 μ) were sliced on the microtome and stained with hematoxylin and eosin. Morphometry of the tumor included evaluation of the volume densities (Vv) of necroses and apoptoses in tumor tissue. Morphometry of the liver was carried out to estimate the volume densities (Vv) of normal liver parenchyma, degenerative hepatocytes, liver parenchyma necroses, and numerical density (Nai) of binuclear hepatocytes (indicator of reparative processes in the liver parenchyma).

The expression of genes involved in the formation of MDR (*mdr1b*, *p53*, *bcl-2*) was evaluated by reverse transcription PCR as follows. Tota cell RNA was isolated from primary culture of RLS_{40} cells, obtained after each course of PCT, by SDS/phenol extraction as described previously [4]. Synthesis of cDNA was then carried out in buffer using summary cellular RNA, randomized hexanucleotide primer, and M-MLV reverse transcriptase (Institute of Chemical Biology and Basic Medicine). Amplification was carried out with pairs of primers synthesized at Technological Laboratory of Institute of Chemical Biology and Basic Medicine.

The sequences of specific primers to *mdr1b*, *bcl-2*, p53 genes and β-actin were selected using OLIGOS software: *mdr1b* (5'-CTGCTGTTGGCGTATTTGGG-3', 5'-TGGCAGAATACTGGCTTCTGCT-3', 170 b. p.), *bcl-2* (5'-TCGCAGAGATGTCCAGTCAGC-3', 5'-CATCCCAGCCTCCGTTATCC-3', 255 b. p.), *p53* (5'-GAACCGCCGACCTATCCTTAC-3', 5'-GTTTGGGCTTTCCTTGAT-3', 412 b. p.), and β-actin (5'-AGCCATGTACGTAGCCATCCA-3', 5'-TCTCCGGAGTCCTCACAATG-3', 81 b. p.).

The PCR products were separated by electrophoresis in 8% PAAG. The resultant band images were processed by computer densitometry (Gel-Pro Analyzer 4.0). In order to evaluate the expression of mRNA, the integral optical density of bands corresponding to gene-specific PCR products was normalized by optical density of β-actin product.

The results were statistically processed using Statistica software, the differences were considered significant at $p<0.05$.

RESULTS

Four passages of the tumor from one mouse to another with subsequent PCT was carried out in order to carry out as many as possible PCT courses modulating tumor cells, which is impossible by carrying out several PCT courses in the same animal because of pronounced toxic effects of drug therapy.

All animals developed a solid tumor node at the site of implantation on day 7. Histologically this node consisted of monomorphic atypical lymphoid cells with frequent mitoses and invasive growth into the hip muscles. The metastases were detected mainly in the liver and were located perivascularly. Necrotic and apoptotic cell death was revealed in tumor tissue

without treatment and after cytostatics. The percentage of apoptotic cells decreased with increasing the number of PCT courses, while the percentage of necroses remained virtually the same: volume density of apoptoses in tumor tissue decreased by 1.7 times after four PCT courses compared to the corresponding parameter that after one PCT course (Fig. 1). Hence, repeated courses of cytostatic therapy reduced tumor cell capacity to apoptosis.

The expression of *mdr1b*, *bcl-2*, and *p53* genes in RLS_{40} cells after cytostatic therapy are presented (Fig. 2). The expression of the studied genes increased with increasing the number of PCT courses. After four PCT courses, the expression of *mdr1b*, *bcl-2*, and *p53* genes increased by 1.3, 2.3, and 1.6 times, respectively, in comparison with the initial values. These data indicate that repeated courses of PCT increased expression of genes associated with the MDR phenotype and hence, reflect the increase in drug resistance of the tumor [3].

Study of the liver morphology showed the development of severe dyscirculatory and destructive changes in the liver of mice with tumors receiving no treatment and after PCT. These changes included centrolobular plethora, discomplectation of cords, sinusoidal collapse, hydropic and balloon protein degeneration of hepatocytes, frequent monocellular and focal necroses. Morphometry revealed changes in the proportion of destructive changes in the liver of experimental animals receiving PCT from one therapeutic course to another. During passage 1 of the tumor (after one PCT course for the tumor), the percentage of degenerative and necrotic cells was about the same (21.9 and 23.1%, respectively), while during passage 4 (four PCT courses for the tumor and only one for the liver) the volume density of necroses in the liver increased by 1.3 times, while that of degenerative cells decreased by 1.4 times in comparison with passage 1 of the tumor (15.5 and 30.6%, respectively), which attests to possible transformation of degeneration into necrosis. The numerical density of binuclear hepatocytes decreased 1.8 times after passage 4 in comparison with passage 1. This attests to significant reduction of the reparative potential of the liver with increasing drug resistance of the tumor even after a single course of cytostatic therapy.

Hence, clonal selection of tumor cells with increase of their drug resistance and hence, reduction of PCT efficiency and more intense growth of the tumor node can increase toxic load to the liver. In other words, the hepatotoxic effects of the tumor increase with increasing its drug resistance. Therefore, severe destructive changes in the liver parenchyma caused by an increase in tumor toxicity augment even after retransplantation of the tumor with subsequent cytostatic treatment, when the tumor is exposed to several PCT courses and the liver to just one course.

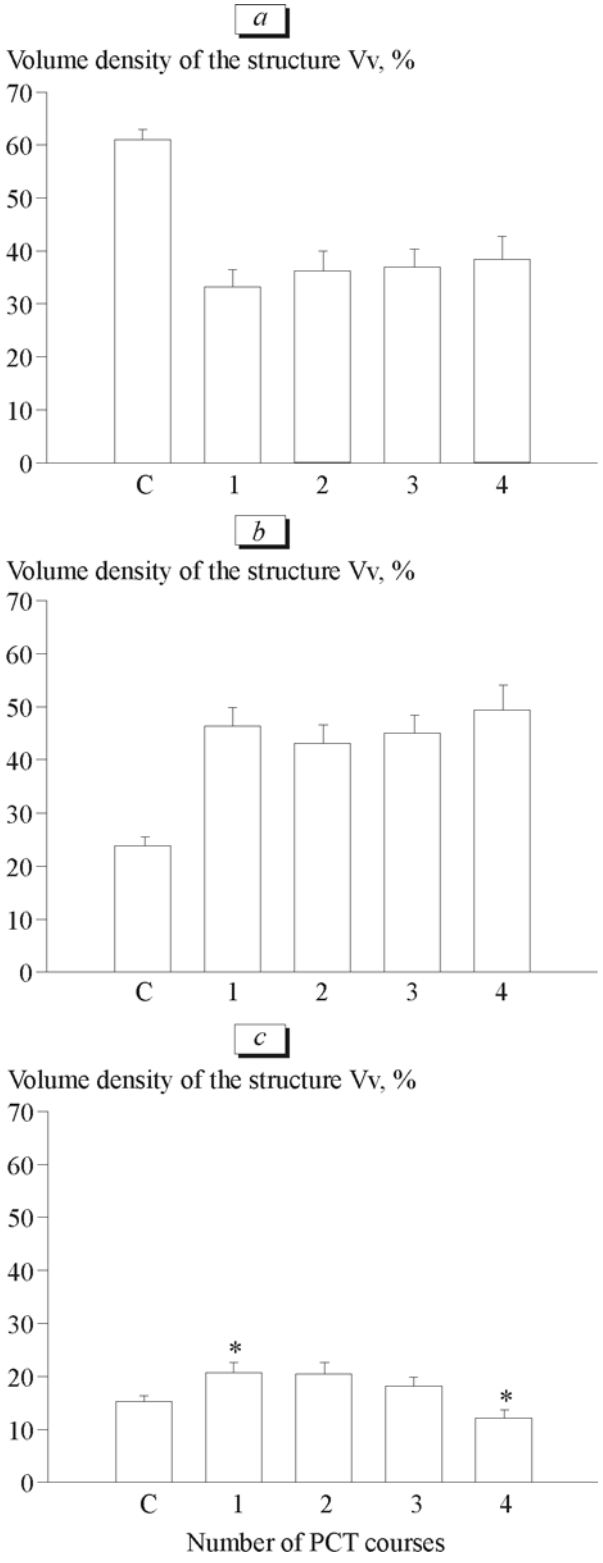

Fig. 1. Morphologic changes in mouse RLS_{40} lymphosarcoma tissue without treatment (C) and after 4 courses of PCT. *Significant differences $p \leq 0.05$. *a*) intact tissue; *b*) necroses; *c*) apoptoses.

Hence, study of methods for control of unjustified toxicity of chemotherapy remains an important problem. One of approaches is individual PCT with

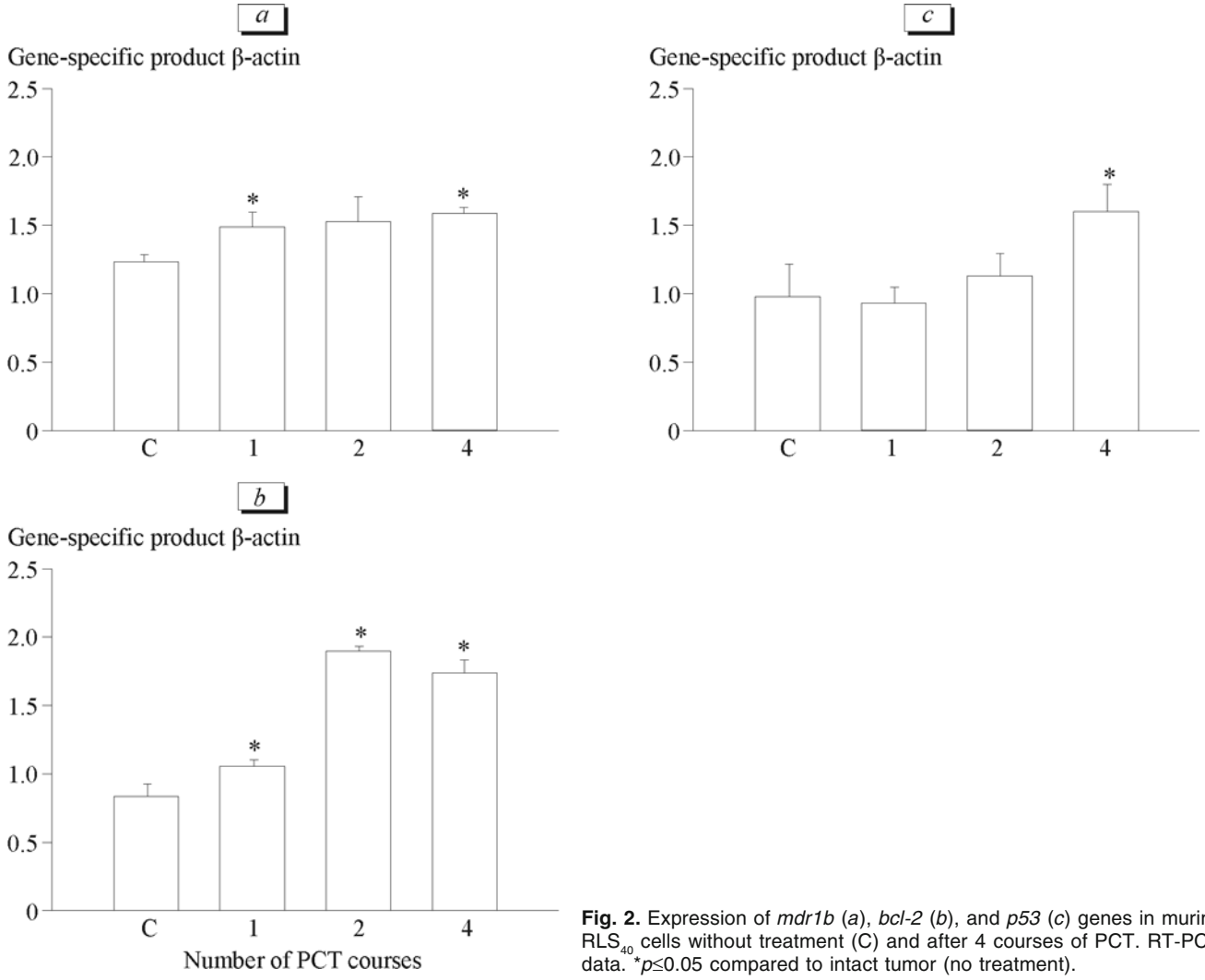

Fig. 2. Expression of *mdr1b* (*a*), *bcl-2* (*b*), and *p53* (*c*) genes in murine RLS_{40} cells without treatment (C) and after 4 courses of PCT. RT-PCR data. $^*p \leq 0.05$ compared to intact tumor (no treatment).

initial evaluation of tumor sensitivity to the standard cytostatic panel in order to rule out the unjustified cytostatic challenge in cases with *a priori* resistant tumor processes.

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