Possible Role of P-Glycoprotein in Cyclophosphamide Resistance of Transplanted Mouse RLS Lymphosarcoma

A. Yu. Grishanova, E. V. Mel'nikova, V. I. Kaledin*, V. P. Nikolin*, and V. V. Lyakhovich

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 139, No. 5, pp. 573-577, May, 2005
Original article submitted December 18, 2004

The causes of different sensitivity of mouse LS lymphosarcoma and its resistant RLS variant to cyclophosphamide were studied. Division of LS and RLS cells stops in the G_2/M phase 24 h after cyclophosphamide treatment, but this stop lasts for more than 48 h in LS cells and less than 24 h in RLS cells. DNA fragmentation, a marker of apoptosis, is observed only in LS cells starting from 24 h after cyclophosphamide treatment. LS and RLS strains do not differ by the expression of *bcl-2*, *bcl-6*, *bax*, *bad*, *mdr1a*, *mdr1b* genes and P-glycoprotein protein. The strains differ by transport activity of P-glycoprotein, tested by SYTO 16 substrate release from cells: activity of P-glycoprotein in RLS cells was 2-fold higher than in LS cells. Presumably, the resistance of RLS tumor to cyclophosphamide-induced apoptosis is a result of inhibition of the apoptotic cascade by P-glycoprotein which is functionally more active in these cells than in LS cells.

Key Words: *cyclophosphamide; mouse lymphosarcoma; resistance; P-glycoprotein; apoptosis*

Drug resistance of tumor cells can be caused by high activity of drug detoxication enzymes in these cells, hyperexpression of genes responsible for drug elimination from cells (transporting genes of multiple drug resistance family: *MDR-1*, *BCRP*, *LRP*, *MRP*), and by changes in genes and proteins regulating apoptosis [3]. Insensitivity to inducion of apoptosis is one of the most important mechanisms of drug resistance of tumor cells. Two main approaches to the induction of apoptotic cell death are known: activation of TNF family receptors with subsequent activation of caspase-8 and caspase-3 and another approach, mediated by ceramide and *Bcl-2* gene family regulating cytochrome C release from mitochondria [10]. The effect of P-glycoprotein (P-gp), product of *MDR1* gene, on these apoptosis mechanisms was revealed for some tumor cells [5,11], though up to recent time P-gp, a member of ATP-dependent transporter family, was traditionally believed to be involved in the formation of resistance to antitumor drugs as a result of their active elimination from the cells [15].

We obtained a continuous mouse LS lymphosarcoma strain highly sensitive to antitumor effect of cyclophosphamide (CP) [2] and its resistant RLS variant [1]. In order to clear out the mechanism of RLS tumor resistance to CP, we investigated the effects of CP on the time course of cell cycle profile and DNA fragmentation in LS and RLS cells and studied constitutive expression of apoptosis inhibitor and promotor genes *bcl-2*, *bcl-6*, *bax*, *bad*, of *mdr1a* and *mdr1b* genes, and expression of P-gp protein and its transport activity.

MATERIALS AND METHODS

The study was carried out on 3-5-month-old male CBA mice from the vivarium of Institute of Cytology and

Institute of Molecular Biology and Biophysics, Siberian Division of Russian Academy of Sciences; *Institute of Cytology and Genetics, Siberian Division of Russian Academy of Sciences, Novosibirsk. ***Address for correspondence:*** agrish@soramn.ru. A. Yu. Grishanova

0007-4888/05/1395-0611

Genetics. The animals were kept under conditions of natural illumination on granulated PK 120-1 fodder (Laboratorsnab). LS and RLS tumors were maintained in ascitic form. Tumor cells were transplanted into hip muscles (10^6 cells/mouse). Each experiment was carried out on 4-5 mice. When the tumor reached 1.5 cm in diameter, the mice were intraperitoneally injected with cyclophosphamide (25 mg/kg; Biokhimik). The animals were sacrificed by cervical dislocation after 8, 24, 48, 72, and 96 h, cell suspension prepared from the tumors was washed with buffered saline (PSB; pH 7.4). For DNA analysis a portion of each sample was fixed in 70% ethanol, for immunofluorescent analysis in 1% paraformaldehyde, for the analysis of mRNA level frozen in liquid nitrogen, for the analysis of P-gp functional activity resuspended in PSB.

Analysis of DNA distribution in cells was carried out on a flow cytofluorometer FACSCalibur (Becton Dickenson) after washing from ethanol and 15-min incubation in PSB with RNase (5 μg/ml) and propidium iodide (20 μg/ml).

Total cell RNA was isolated as described previously [6]. DNase treatment was carried out using DNase (Promega). The level of specific mRNA was evaluated by semiquantitative reverse transcription PCR. Reverse transcriptase (Sibenzyme) was used for *in vitro* cDNA synthesis. Amplification was carried out with pairs of primers synthesized on ASM-800 (Biosset Firm). Primer sequences for *bad* were taken from EMBL base (direct 5'-ATCGCTGTGTCCCTTTA-3', reverse 5'-CCCCAGTTATGACAGGACAG-3', annealing temperature (Tm) 63°, product 227 b. p.). Sequences of primers specific to nucleotide sequences of other genes were selected using Oligo software: *bcl-2* (5'-TTGATCTTCTTTCGGCCTGT-3', 5'-CATCCTCCCATTTAAGACACTC-3', Tm 63°, 332 b. p.), *bcl-6* (5'-TCCTATGGTGCTGACCCTACT-3', 5'-TGAGCGACAGACACTTGGAT-3', Tm 63°, 323 b. p.), *bax* (5'-TTTTTGCTACAGGGTTTCATCC-3', 5'-GCCTCAGCCCATCTTCTTCC-3', Tm 66°, 510 b. p.), *mdr1a* (5'-GTCAGCATCCCACATCATCA-3', 5'-TGCCCTCACAATCTCCTCAT-3', Tm 63°, 436 b. p.), *mdr1b* (5'-CCCAGGAGCCCATTCTCTTT-3', 5'-TTGCCGTTCTCAATCACCAC-3', Tm 60°, 400 b. p.), β-actin (5'-GACGGGGTCACCCACACTGT-3', 5'-GAGTACTTGCGCTCAGGAGGAG-3', Tm 65°, 545 b. p.). Each sample was amplified twice. PCR products were analyzed after electrophoresis in 2% agarose gel, staining with ethidium bromide, scanning by DNA Analyzer videosystem, and densitometried using Total Lab software.

The expression of P-gp on the cell surface was analyzed on a flow cytofluorometer after indirect immunofluorescent staining of cells washed from the fixative using primary antibodies c-19 to mouse P-gp (Santa Cruz) and second antibodies to goat globulins, labeled with FITC (Santa Cruz). The expression was evaluated as the ratio of the mean fluorescence intensity of cells treated with antibodies to P-gp to mean fluorescence intensity of isotypical control [9].

Functional activity of P-gp was studied using 0.03 μM fluorescent substrate SYTO-16 (Molecular Probes) with or without 500 μM verapamil as the inhibitor [9]. Functional activity was evaluated on a flow cytofluorometer. Activity of P-gp was evaluated by SYTO-16 release, evaluated as the ratio of the mean fluorescence intensities of cells with and without verapamil.

The results were statistically processed using Statistica software. The significance of differences was evaluated by the Kolmogorov—Smirnov test.

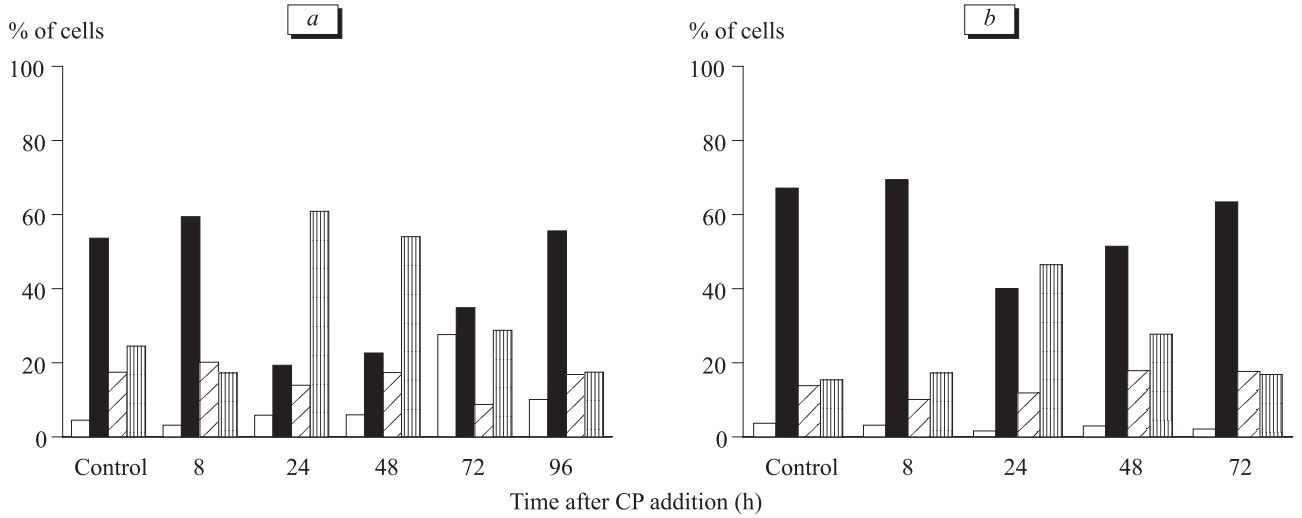

Fig. 1. Dynamics of LS (*a*) and RLS lymphosarcoma cell (*b*) distribution by cell cycle phases under the effect of cyclophosphamide (CP). Cell populations in the G_0/G_1 phase: dark bars; S: cross-hatched bars; G_2/M phase of the cell cycle: vertical hatched bars; cells with DNA content $<G_0/G_1$: light bars.

RESULTS

Cyclophosphamide induced accumulation of LS and RLS cells in the G_2/M phase of the cell cycle after 24 h, and the percentage of cells in the G_2/M phase after 24 h was the same for LS and RLS strains (Fig. 1), but the arrest of LS cells in the G_2/M phase persisted after 48 and 72 h, while in RLS cells it virtually disappeared by the 48th hour.

Apoptosis-inducing effect of CP was evaluated by analyzing cells with hypodiploid content of DNA (sub-G_o/G_1 or $<G_o/G_1$; Fig. 1). The percentage of LS cells with fragmented DNA appreciably increased by the 24th hour after CP addition and was retained at this level during the entire period of observation, which confirms electrophoresis data on DNA fragmentation [2]. The percentage of RLS cells with fragmented DNA

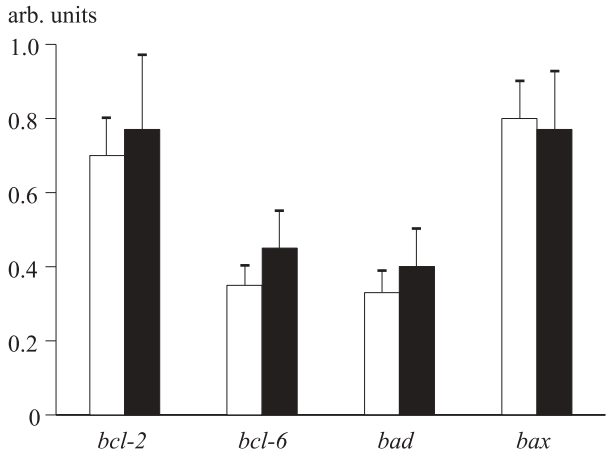

Fig. 2. Relative content of apoptosis factors *bcl*-2, *bcl-6*, *bax*, and *bad* mRNA in LS and RLS lymphosarcoma cells. The ratio of optical density of genes RT-PCR products to optical density of β-actin gene product is taken for the arbitrary unit. Light bars: LS; dark bars: RLS.

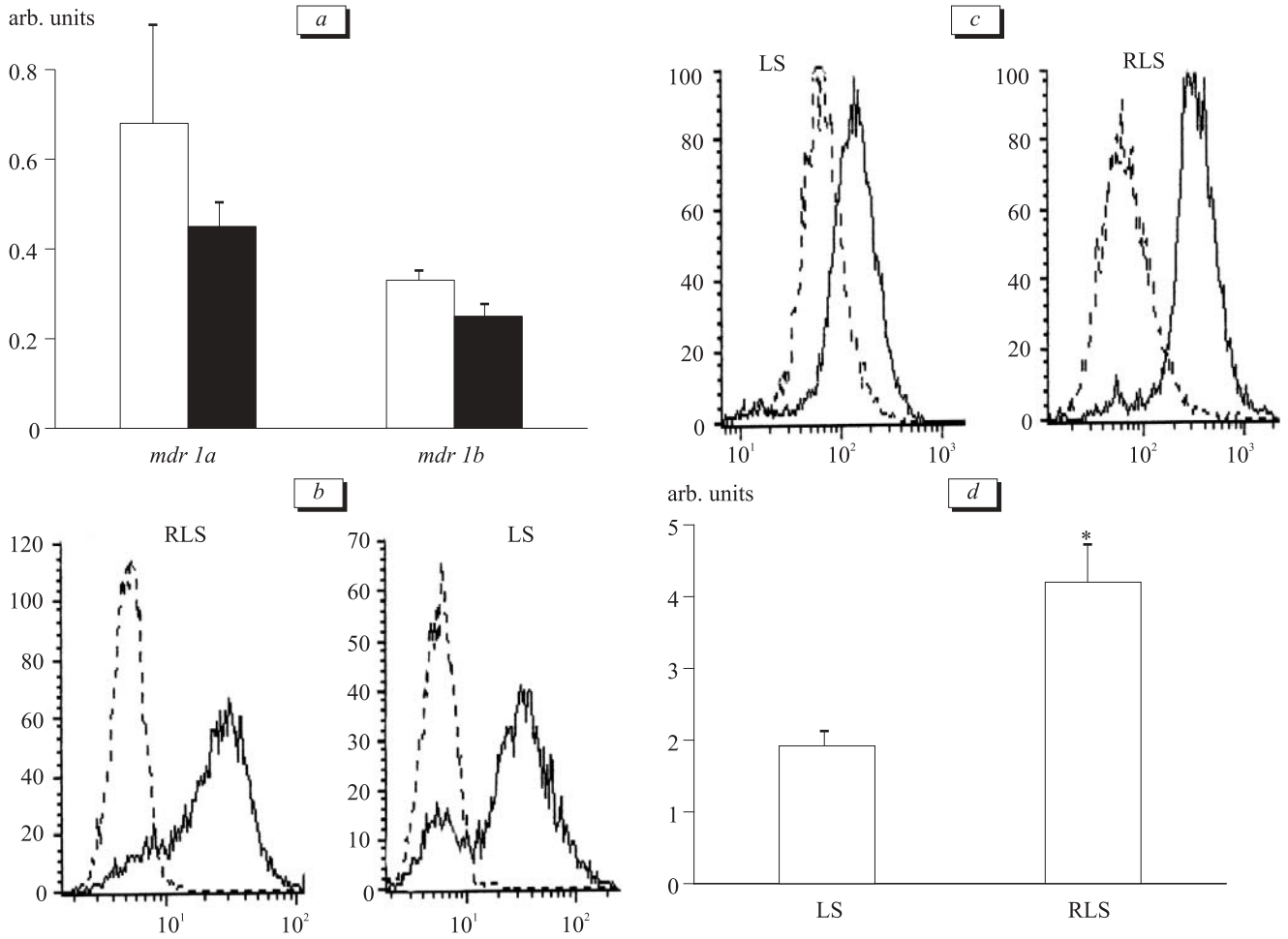

Fig. 3. Expression of genes encoding P-glycoprotein (*a*), expression of P-glycoprotein protein (*b*), and P-glycoprotein activity (*c*, *d*) in LS and RLS lymphosarcoma cells. *a*) relative content of *mdr1a* and *mdr1b* mRNA. The ratio of optical density of genes RT-PCR products to optical density of β-actin gene product is taken for the arbitrary unit; *b*) fluorescence intensity of LS and RLS cells after staining with antibodies to P-glycoprotein and second antibodies (continuous line) and treatment with second antibodies alone (intermittent line); *c*) SYTO-16 fluorescence intensity in LS and RLS cells in the presence of verapamil inhibitor (continuous line) and without it (intermittent line). *b*, *c*: abscissa: fluorescence intensity (arb. units); ordinate: cell number. *d*: activity of P-glycoprotein determined by SYTO-16 release and estimated as the ratio of mean fluorescence intensities of cells with and without verapamil. *$p<0.001$ compared to LS.

did not change over 96 h, which can indicate the absence of apoptosis induction in these cells under the effect of CP.

Hence, LS and RLS cells differ by the duration of block in the G_2/M phase, caused by CP, and by sensitivity to apoptosis induction.

Bcl-2 family genes play a central role in apoptosis regulation. Members of this gene family — inhibitors and promotors of programmed cell death — regulate the formation of channels in the mitochondrial membrane by modulating the release of cytochrome C and apoptosis-inducing factor into the cytoplasm from the mitochondria. In order to evaluate the role of apoptosis factors in the mechanism of RLS strain resistance, we studied the expression of apoptosis inhibitors (*bcl-2* and *bcl-6*) and inductors (*bax* and *bad*) genes in LS and RLS cells. Gene expression in cells of these tumors virtually did not differ (Fig. 2).

No differences in the levels of *mdr1a* and *mdr1b* mRNA in LS and RLS cells were detected (Fig. 3, *a*). The expression of P-gp on cell surface also did not differ (Fig. 3, *b*): the ratio of fluorescence intensity of cells treated with anti-P-gp antibodies to fluorescence intensity of isotypical control was 4 in LS and 4.2 in RLS cells. However, functional activity of P-gp in RLS cells evaluated by SYTO-16 substrate release, was 2-fold higher than in LS cells (Fig. 3, *c*, *d*).

Cyclophosphamide is not a P-gp substrate [12], and hence, RLS cell resistance is not formed at the expense of CP release from cells. Presumably, functional activity of P-gp is related to apoptosis resistance of RLS. P-gp can transport phospholipids [11] and thus can reduce the pool of sphingomyelin located on the inner plasma membrane; sphingomyelin participates in the formation of intracellular ceramide, the key proapoptotic molecule of apoptosis signal cascade [8]. P-gp is involved in ATP transport [9] and pH modification inside the cell towards the alkaline values [14]. These cytosol factors can be modulators of apoptosis [7,13]. Therefore, activation of P-gp transport function can modulate the intracellular level of ceramide, ATP, and/or pH and eventually lead to apoptosis inhibition.

Hence, the resistance of RLS lymphosarcoma strain to CP is caused by its insensitivity to apoptosis induction. Presumably, high functional activity of P-gp, characteristic of this tumor, is one of the causes of this insensitivity.

REFERENCES

1. A. Yu. Grishanova, V. I. Kaledin, T. V. Zueva, *et al.*, *Biomed. Khim.*, **49**, 27-34 (2003).
2. V. I. Kaledin, V. P. Nikolin, T. A. Ageeva, *et al.*, *Vopr. Onkol.*, **46**, 588-593 (2000).
3. A. A. Stavrovskaya, *Biokhimiya*, **65**, 112-126 (2000).
4. E. H. Abraham, A. G. Prat, L. Gerweek, *et al.*, *Proc. Natl. Acad. Sci. USA*, **90**, 312-316 (1993).
5. C. Bezombes, N. Maestre, G. Laurent, *et al.*, *FASEB J.*, **12**, 101-109 (1998).
6. P. Chomczynski and N. Sacchi, *Anal. Biochem.*, **162**, 156-159 (1987).
7. Y. Eguchi, A. Srinivasan, K. Tomaselli, *et al.*, *Cancer Res.*, **59**, 2174-2181 (1999).
8. Y. A. Hannun, *Science*, **274**, 1855-1865 (1996).
9. S. Huet, J. P. Marie, N. Gualde, *et al.*, *Cytometry*, **15**, 248-256 (1998).
10. X. Jiang and X. Wang, *Annu. Rev. Biochem.*, **73**, 87-106 (2004).
11. R. W. Johnstone, A. A. Ruefli, K. M. Tainton, and M. J. Smyth, *Leuk. Lymphoma*, **38**, 1-11 (2000).
12. C. J. Matheny, M. W. Lamb, K. L. R. Brouwer, and G. M. Pollack, *Pharmacotherapy*, **21**, 778-796 (2001).
13. A. Rebollo, J. Gomej, A. Martinez de Aragon, *et al.*, *Exp. Cell Res.*, **218**, 581-585 (1995).
14. P. D. Roepe, L. Y. Wei, J. Cruz, and D. Carlson, *Biochemistry*, **32**, 11,042-11,056 (1993).
15. A. van Helvoort, A. J. Smith, H. Sprong, *et al.*, *Cell*, **87**, 507-517 (1996).