Cyclophosphamide Metabolite Inducing Apoptosis in RLS Mouse Lymphosarcoma Cells Is a Substrate for P-Glycoprotein

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RLS lymphosarcoma characterized by enhanced expression of *mdr1a* and *mdr1b* genes encoding P-glycoprotein is insensitive to low doses of cyclophosphamide, but is susceptible to its high doses approximating the maximum tolerated doses. Induction of apoptotic death of RLS cells by high doses of cyclophosphamide was demonstrated by cytofluorometry and electrophoresis. Experiments on RLS_{40} tumor cells derived from RLS lymphosarcoma and characterized by more intensive expression of *mdr1a/1b* genes showed that the therapeutic effects of cyclophosphamide increased under conditions of simultaneous suppression of these genes by specific small interfering RNA (siRNA). These findings suggest that active cyclophosphamide metabolite can be a substrate for P-glycoprotein.

Key Words: *RLS tumor; cyclophosphamide; P-glycoprotein; apoptosis*

We previously obtained transplantable murine lymphosarcoma LS highly sensitive to the therapeutic effect of cyclophosphamide (CP) and its daughter strain RLS exhibiting resistance to low doses of cyclophosphamide [1-3,6]. Despite different sensitivity to apoptosis induction, these tumors did not differ by the expression of proapoptotic (*bax* and *bad*) and antiapoptotic (*bcl-2* and *bcl-6*) genes. However, the expression of *mdr1a/1b* genes belonging to ABS transporter gene family in RLS tumor cells 2.5-fold surpasses that in the parent LS cells [1,13]. P-glycoprotein, a product of *mdr1a/1b* genes, acts as a transmembrane pump and participates in the formation of the multiple drug resistance phenotype due to active drug elimination from tumor cells. However, the expression of *mdr1a/1b* genes belonging to ABS transporter gene family in RLS tumor cells 6-fold surpasses that in the parent LS cells [13]. However, during repeated *in vivo* passages of the tumor, bcl-2^+ phenotype was lost and the expression of this gene returned to the level observed in LS cells.

CP metabolites were never mentioned as the substrates of P-glycoproteins [5,12,14]. However, analysis of the expression profiles of pro- and antiapoptotic genes and ABC-transporters family genes in RLS cells drove us to an assumption that tumor resistance to apoptotic and probably antitumor effects of CP can be explained by rapid P-glycoprotein-mediated efflux of CP metabolite from tumor cells.

The aim of the present study was to analyze the effects of high doses of CP on the growth of RLS tumor *in vivo*, to evaluate the capacity of CP to induce apoptotic death of tumor cells, and to find out whether CP metabolite is a substrate for P-glycoprotein. To find out whether down-regulation of P-glycoprotein

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expression in the tumor enhances the therapeutic antitumor effect of CP, we studied the effect of combined treatment with CP and specific mdr1a/1b siRNA against *mdr1a* and *mdr1b* gene RNA on RLS_{40} derived from RLS tumor and characterized by more intensive expression of *mdr1a* and *mdr1b* genes.

MATERIALS AND METHODS

The study was performed on 4-month-old male CBA mice obtained from Nursery of Institute of Cytology and Genetics, Siberian Division of Russian Academy of Sciences. LS and RLS tumors are maintained in the vivarium in ascitic form on these mice.

Tumor cells (2×10^6 LS cells and 2×10^5 RLS cells) were transplanted to CBA mice into the thigh muscles (for the formation of a solid tumor) or intraperitoneally (for the formation of ascitic tumor). On day 10 after tumor transplantation, CP (cyclophosphan-LANS) was injected intraperitoneally in a dose of 20-50 mg/kg (to mice with LS) or 200 mg/kg (to mice with RLS). Animal life-span was recorded throughout the experiment. Forty-eight hours after CP injection, 3 mice with solid tumors from each group were sacrificed, the tumors were isolated, homogenized, and the homogenates were pooled within the group. DNA was isolated from pooled homogenates as described elsewhere [7] and analyzed in 1.4% agarose gel.

Forty-eight hours after CP injection, 3 mice with ascitic tumors from each group were sacrificed and ascitic fluid was collected. The cells were pelleted by centrifugation, washed, and stained with popNexin FITC apoptosis detection kit (Chemicon) according to manufacturer's instructions. The cells were analyzed by flow cytofluorometery on FC500 cytometer (Beckman Coulter).

Small interfering RNA (siRNA) were synthesized by the phosphate amide method at the Laboratory of RNA Chemistry (Institute Chemical Biology and Fundamental Medicine, Siberian Division of the Russian Academy of Sciences). We used mdr1a/1b siRNA to *mdr1a* and *mdr1b* gene RNA: GGCUGGACAAGCUGUGCAUGG (sense) and AUGCACAGCUUGUCCAGCCAA (antisense). *luc* siRNA to luciferase gene mRNA served as the control: CGUUAUUUAUCGGAGUUGCAG (sense) and GCAACUCCGAUAAAUAACGCG (antisense). For obtaining siRNA duplexes, the reaction mixture containing antisense and sense sequences, 30 mM HEPES-KOH, 100 mM KAc, and 2 mM MgAc was incubated at 90°C for 1 h and then slowly cooled to 37°C for 1 h.

RLS_{40} tumor (2×10^5 cells) was transplanted intraperitoneally to CBA mice and after 10-12 days the siRNA duplexes with lipofectamine in Opti-MEM medium were intraperitoneally injected; lipofectamine in Opti-MEM medium served as the control. Four hours after the injection, the mice were sacrificed, ascitic fluid was collected, pooled within the groups, and the obtained cell suspension was transplanted intramuscularly (2×10^5 cells, 0.1 ml per mouse, 20 mice per group) for induction of solid tumors. A part of RLS_{40} cells was used for isolation of total RNA by the method of SDS/phenol extraction [8]. Two days after transplantation the mice were divided into 2 subgroups intraperitoneally receiving 200 mg/kg CP and physiological saline, respectively. Tumor diameter was measured with a caliper throughout the experiment.

Expression of *mdr1a* and *mdr1b* genes was evaluated by reverse-transcription PCR; the primers and reaction conditions were described previously [13]. β-Actin mRNA was used as the internal standard. PCR products were separated by electrophoresis in 8% PAAG under native conditions. The bands were densitometried using Gel-Pro Analyzer 4.0. For evaluation of mRNA expression, the integral optical density corresponding to gene-specific PCR products were standardized by optical density of β-actin product.

The data were processed statistically, the differences were evaluated using Student's *t* test.

TABLE 1. Effect of CP on Life-Span of Tumor-Bearing Animals and Induction of Apoptosis in LS and RLS cells

Tumor type	CP, mg/kg	Mean life-span, days[2]	Mean life-span prolongation, %	Percent of apoptotic tumor cells[3]
LS[1]	-	15.2±0.7	-	5.7
	20	24.6±1.5**	61.8	24.5±1.7**
RLS[1]	-	13.5±0.3	-	5.7±0.9
	200	16.5±0.5**	22.2	16.4±4.1*

Note. "–": control (untreated tumor-bearing mice). *$p<0.05$, **$p<0.001$ in comparison with the control. [1]LS (2×10^6 cells per mouse) or RLS (2×10^5 cells per mouse) were intraperitoneally transplanted to male CBA mice for the formation of ascitic tumor. On day 10 after tumor transplantation, CP was injected in a dose of 20 mg/kg (to mice with LS) or 200 mg/kg (to mice with RLS). [2]The data are presented as Mean±SEM. [3]Flow cytofluorometry data; at least 15,000 events were analyzed in each sample.

RESULTS

We studied the effect of high doses of CP on the life-span of tumor-bearing animals and the capacity to induce apoptotic death of tumor cells (Table 1). To this end, RLS cells (2×10^5/mouse) were intraperitoneally or intramuscularly transplanted to male CBA mice for the formation of ascitic and solid tumors. LS cells (2×10^6/mouse) were transplanted as the positive control, because LS is known to be sensitive to CP treatment. After 10 days, the mice with RLS tumors received single intraperitoneal injection of CP in a dose of 200 mg/kg; mice with LS received 20 mg/kg CP. Forty-eight hours after CP injection, cells from ascitic fluid were isolated, stained with annexin V, and the number of apoptotic cells was evaluated by flow cytofluorometry. It is known that annexin V irreversibly binds to phosphatidylserine, which is normally localized on the inner layer of the membrane, but appears on the outer layer after induction of apoptosis [9,10]. Expression of phosphatidylserine on the outer membrane surface is a sign of apoptosis and is observed starting from the early stages to complete cell degradation. It was found that the number of apoptotic cells after treatment with 200 mg/kg CP increased 3 fold in comparison with the control (untreated tumor-bearing mice). The life-span of tumor bearing mice receiving CP increased by 22.2%. As expected, the effect of CP treatment was more pronounced in case of LS tumors: animal life-span increased by 61.8%. Analysis of DNA integrity in tumor cells obtained from solid RLS and LS tumors after CP treatment revealed DNA fragmentation in RLS cells typical of apoptosis and similar to that observed in LS cells (Fig. 1).

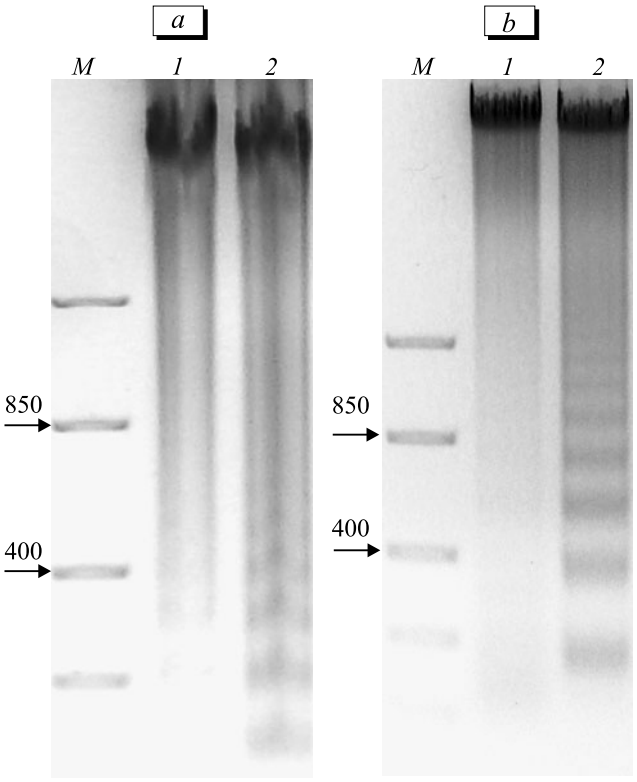

Fig. 1. Analysis of DNA from LS (*a*) и RLS (*b*) tumor cells; 1.4% agarose gel stained with ethidium bromide. *M*: FastRules Low Range marker (Fermentas); *1*) DNA isolated from LS or RLS tumor cells from untreated animals; *2*) tumor cell DNA isolated 48 h after CP injection in doses of 20 mg/kg (LS) and 200 mg/kg (RLS).

We previously showed that CP in a dose of 25 mg/kg inhibits the growth of solid LS tumor and had no effect on the growth of solid RLS tumor [3]. Cytophotometry showed that after CP treatment the number of cells with haploid DNA content (indicator of apoptosis) in LS tumor increased by more than 6-times (from 4.5 to 27.8%), while in RLS tumor this parameter remained at the control level (<3%) [1]. It was previously demonstrated that CP in a dose of 150 mg/kg inhibits the growth of RLS tumor by 20% and 2-4-fold increases activity of cathepsins B, L and D in tumor cells, which indirectly attests to induction of apoptosis. In the present study we directly proved that CP in a dose producing pronounced therapeutic effect induces apoptotic death of RLS cells (Table 1, Fig. 1).

Thus, CP induced apoptotic death of RLS tumor cells, but in this case its dose was 10-fold higher than for LS tumor [3]. Elucidation of the mechanisms underlying the differences in CP doses triggering apoptosis in tumor cells was one of the tasks of our study. Inefficiency of low doses of CP in case of RLS can be related to hyperexpression of *mdr1a* and *mdr1b* genes in tumor cells. This leads to the efflux of active CP metabolite from cells and it does not accumulate in cells in amounts sufficient for triggering apoptosis. Ten-fold increase in CP dose probably results in an increase in its concentration in cells and hence to the increase in the number of apoptotic cells. This agrees with a previous study [6] demonstrating significant enhancement of the therapeutic effect of CP on RLS tumor after increasing its dose from 150 to 200 mg/kg.

In a previous study [13], selection of the primary culture of RLS tumor cells on increasing concentration of vinblastine yielded RLS_{40} cells characterized by enhanced expression of *mdr1a* and *mdr1b* genes. We used this tumor to find out, whether its sensitivity to CP is restored without increasing CP dose under conditions of simultaneous suppression of *mdr1a* and *mdr1b* gene expression. To this end we used mdr1a/1b siRNA to *mdr1a* and *mdr1b* gene RNA. This siRNA suppresses expression of both genes due to high homology of *mdr1a* and *mdr1b* genes. *luc* siRNA to luciferase gene mRNA served as the control.

siRNA was injected intraperitoneally to mice with ascitic RLS_{40} tumor and after 4 h the tumor cells were

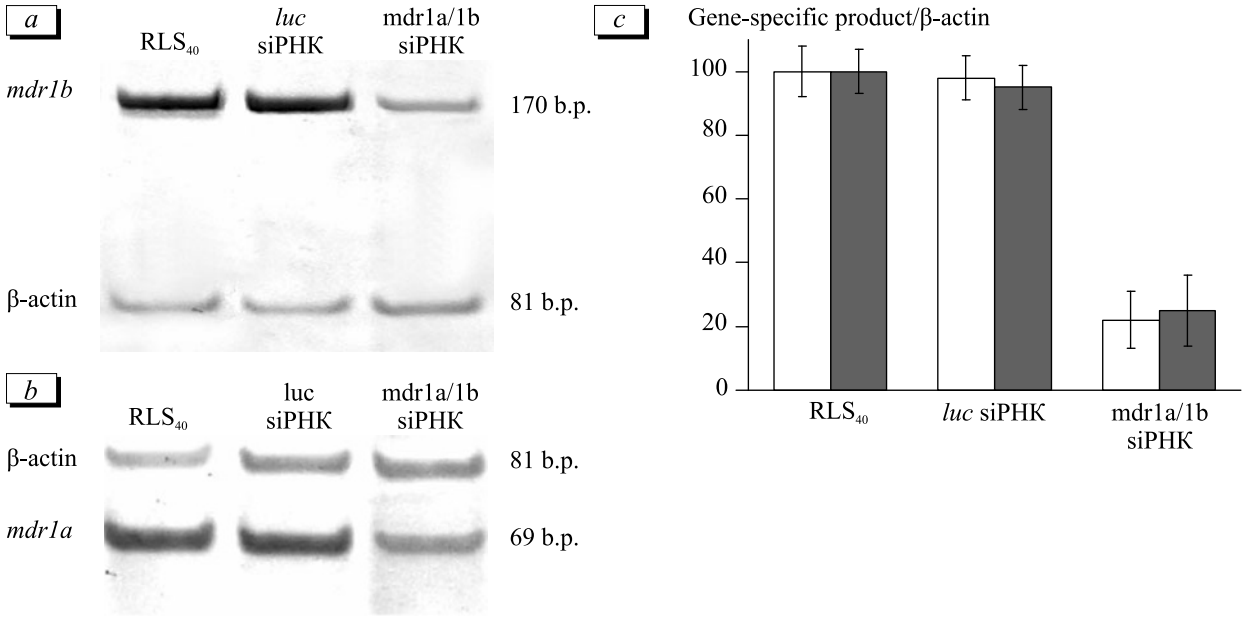

Fig. 2. Suppression of *mdr1b* (*a;* open bar) and *mdr1a* (*b;* dark bar) gene expression in RLS_{40} tumor cells *in vivo* tumor cells after treatment with mdr1a/1b siRNA. *c*) quantitative processing of the data presented on *a* and *b*. Experimental error did not exceed 10%.

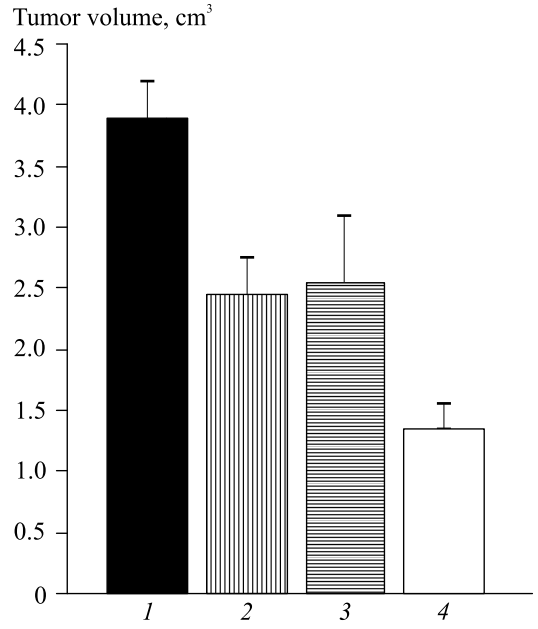

Fig. 3. Effect of combined therapy with mdr1a/1b siRNA and CP on the growth of RLS_{40} tumor in animals. *1*) without CP treatment; *2*) 200 mg/kg ЦФ; *3*) control luc siRNA in a dose of 15 μg per mouse and CP in a dose of 200 mg/kg; *4*) mdr1a/1b siRNA and 200 mg/kg CP. The data on tumor volume correspond to day 14 after transplantation of tumor cells.

inoculated intramuscularly to healthy animals for the formation of solid tumor. Two days after transplantation, the mice received 200 mg/kg CP or physiological saline (control) Total RNA was isolated from RLS_{40} tumor and the expression of *mdr1a* and *mdr1b* genes was evaluated by PCR. We demonstrated changed expression of *mdr1a* and *mdr1b* genes in RLS_{40} tumor cells (Fig. 2). Transfection of tumor cells with mdr1a/1b siRNA *in vivo* (injection into the ascitic tumor) led to a 5-fold decrease in the expression of both *mdr1b* and *mdr1a* genes in comparison with the control (tumor without treatment and tumor treated with *luc* siRNA). Figure 3 shows tumor size in animals receiving combined treatment with mdr1a/1b siRNA and CP. The growth of tumors from non-transfected and *luc* siRNA-transfected RLS_{40} cells under the effect of CP was inhibited by 37.2 and 34.4%, whereas inhibition of the tumor growth of from cells transfection by *mdr1a/1b* siRNA was 2-fold more effective and constituted 66.7% (Fig. 3).

It is known that apoptosis of tumor cells is triggered by not CP, but active CP derivatives formed in the liver, unstable metabolite 4-hydroxycyclophosphamide and phosphoramide mustard formed after its degradation [4,11]. CP metabolites were never mentioned as the substrates of P-glycoproteins [5,12,14]. However, our experiments showed that the resistance of RLS_{40} and RLS tumors to CP is related directly to hyperexpression of P-glycoprotein determining the multiple drug resistance phenotype. Thus, we can conclude that active CP metabolites are substrates for P-glycoprotein.

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