BIOPHYSICS AND BIOCHEMISTRY

Effect of Nitric Oxide Donor, a Modulator of Tumor Drug Resistance, on Cell Death and p53 Protein Expression

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Exogenous NO donor 3,3-bis-(nitroxymethyl)oxetane (NMO) was synthesized at the Institute for Problems of Chemical Physics (Russian Academy of Sciences). This compound was shown to inhibit cell death (apoptosis and necrosis) in cyclophosphamide-sensitive and cyclophosphamide-resistant P388 murine tumor. p53 protein was expressed in both lines of tumor cells. NO donor NMO had little effect on p53 protein expression in cells of both stains. Our results suggest that the proapoptotic effect of NMO is mediated by the p53-independent molecular mechanisms.

Key Words: *drug resistance; NO donors; apoptosis; p53 protein expression*

NO is involved in a variety of signal pathways. Moreover, NO plays a role in the development of neoplasms due to dysfunction of these pathways. Previous studies showed that NO contributes to the effect of chemotherapeutic agents [5,8, 12]. It was hypothesized that NO plays a role in tumor drug resistance. The expression of two NO synthases in adriamycin-resistant MCF-7 cells was 2-fold lower than in sensitive cell line [7]. Administration of NO donors had a delayed effect on the development of drug resistance in two lines of cultured tumor cells [9]. A correlation was found between the induction of multidrug resistance and reduction of NO synthesis [10]. Published data show that statins reverse doxorubicin resistance in human malignant mesothelioma cells and this effect is realized via NO [11]. NO donor was more potent that standard drugs on the model of cisplatin-resistant human ovarian cancer [2].

Previous experiments showed that NO is involved in the process of apoptosis. Functional activity of NO is related to p53 protein, which plays a key role in adaptation of cells (*e.g.*, tumor cells) to stress factors and treatment with chemotherapeutic agents [1,13,15].

Our previous studies showed that NO donor 3,3-bis-(nitroxymethyl)oxetane (NMO) had a delayed effect on the development of cyclophosphamide resistance in P388 murine leukemia cells. Moreover, this compound increased the efficacy of cytostatic therapy [12]. Here we studied the effect of NMO on cell death (apoptosis and necrosis) and p53 protein expression.

MATERIALS AND METHODS

Experiments were performed on P388 murine leukemia cells and its cyclophosphamide-resistant va-

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riant (P388/CP). The strains were maintained by intraperitoneal transplantation (10^6 cells per mouse) at 8-10-day intervals. NMO [6] was dissolved in sterile distilled water and injected intraperitoneally to mice (single dose 9.5 mg/kg).

Morphological study of cell death was performed as described elsewhere [3]. A suspension of living ascitic cells (100 ml) was stained with acridine orange and ethidium bromide (100 mg/ml, Sigma Chem. Co) for 10 min. The early and late stages of apoptosis and necrosis were evaluated from the intensity of staining. The ratio of dead cells was estimated under a Leitz Diaplan microscope equipped with a fluorescence attachment. Five hundred cells were counted in each sample.

Ascitic fluid was obtained from mice to study p53 protein expression. Ammonium chloride was added to destroy erythrocytes. The mixture was centrifuged at 500*g* for 3 min. The cells were lysed in RIPA buffer, which consisted of 500 mM HEPES, 150 mM NaCl, 1 mM EDTA, 25 mM NaF, 10 mM $ZnCl_2$, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, and 1 mM phenylmethylsulfonyl fluoride (PMS). Cell lysates were isolated by centrifugation at 10,000*g* for 15 min. Protein concentration in lysates was measured by the method of Lowry. Denaturing electrophoresis of proteins was performed in 10% polyacrylamide gel. Cell lysate proteins (20 mg) were applied to each band. After electrophoresis, proteins were put on a Hybond-C nylon membrane (Amersham). Immunoblotting was performed with rabbit anti-p53 antibodies (Santa Cruz) and horseradish peroxidase-conjugated goat antibodies to rabbit immunoglobulin G (Amersham).

The results of immunoblotting assay were analyzed by means of Gel Analysis 1.0 software. The significance of differences was estimated by Student's *t* test. The significance level was 95%.

RESULTS

In vivo morphological study was performed to evaluate the effect of NMO on cell death in P388 and P388/CP tumors.

Table 1 shows that 10-12% tumor cells of both strains die in control animals. Apoptotic and necrotic cells were found in both strains. Tumor cells death was shown to increase 1 h after administration of NMO. These changes were particularly pronounced in the sensitive strain. The number of apoptotic cells in strains P388 and P388/CP was elevated by 4 and 2.5 times, respectively. Treatment with NMO was followed by an increase in the number of necrotic cells in both strains. The intensity of necrosis was higher in P388 leukemia cells than in P388/CP cells (increase by 4 and 2.8 times, respectively).

Apoptosis can be realized via various mechanisms, but p53 protein usually plays a key role in this process. The direct effect of NO on p53 protein is related to tyrosine nitration and binding of the zinc atom in the DNA-binding protein domain [14]. The indirect effect of NO is associated with activation of protein kinase p38 and MAP-kinase cascade [4]. When NO does not affect the expression of p53 protein, its influence is mediated by the p53-independent mechanisms. Phosphorylation of p53 protein is accompanied by its accumulation and activation. p53-dependent apoptosis determines the development of tumor drug resistance to various chemotherapeutics. We studied the effect of NMO on p53 protein expression in P388 leukemia cells of cyclophosphamide-sensitive and cyclophosphamide-resistant strains.

The expression of p53 protein was high in cells of the parent and cyclophosphamide-resistant strains of P388 leukemia (Figs. 1 and 2). Small differences in p53 protein expression were found between these strains. These data suggest that the development of cyclophosphamide resistance is not related to p53 dysfunction. Individual differences in p53 protein expression were revealed in control and NMO-treated cells (Figs. 1 and 2). Moreover, the degree

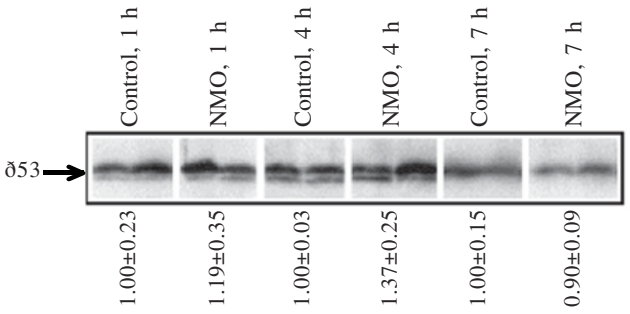

Fig. 1. p53 expression in P388 leukemia cells 1, 4, and 7 h after administration of NMO. Here and in Fig. 2: relative optical density of p53 bands is shown by numbers (immunoblotting). Optical density of p53 bands in control samples is taken as one unit.

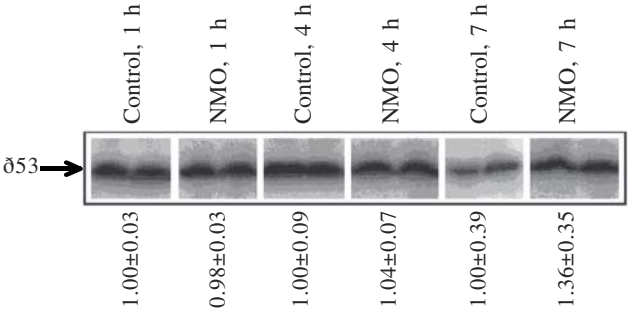

Fig. 2. p53 expression in P388/CP cells 1, 4, and 7 h after administration of NMO.

TABLE 1. Tumor Cell Death 1 h after *in Vivo* Treatment with a NO Donor NMO

Preparation	P388 (parent strain)		P388/CP (cyclophosphamide-resistant strain)	
	apoptosis, %	necrosis, %	apoptosis, %	necrosis, %
Control	4.1±0.2	5.6±0.8	7.6±0.4	4.5±0.6
NMO	16.2±0.5	22.3±1.4	19.3±0.4	12.8±0.7

of p53 expression in P388 leukemia cells was modified in time. The observed changes are probably related to diurnal variations in physiological activity of tumor cells. Temporal changes were not found in cells of the resistant tumor.

NMO had a modulatory effect on p53 protein expression. The increase in p53 protein expression in cells of the resistant and sensitive leukemia strain was particularly pronounced by the 4th and 7th hour after NMO administration. As differentiated from the resistant strain, p53 level in cells of the sensitive strain decreased by the 7th hour after NMO administration. It should be emphasized that the observed changes in p53 protein expression were insignificant (as compared to the control) and did not exceed the standard deviation limits.

Our results indicate that the induction of cell death after treatment with NO donor NMO is accompanied by minor changes in p53 protein expression in the sensitive and resistant strains of leukemia P388. It may be suggested that NO does not activate the mechanisms of p53 induction in cells of the test strains. NMO-induced cell death does not depend on functional activity of p53 protein. We showed for the first time that the studied NO donor can induce apoptosis via a p53-independent mechanism. Further studied are required to test this hypothesis.

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