NO-Generating Compounds Modify Tumoritoxic Effect of Doxorubicin

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The effects of NO-generating compounds on the tumoritoxic effect of doxorubicin was evaluated by changes in DNA synthesis and apoptotic death of Ehrlich adenocarcinoma cells *in vitro*. NO donors significantly inhibited the cytotoxic effect of doxorubicin, which manifested in activation of DNA synthesis and decreased induction of apoptosis. Presumably, NO acts a factor protecting tumor cell DNA from the damaging effect of doxorubicin.

Key Words: *Ehrlich carcinoma; doxorubicin; nitrogen oxide; proliferation; apoptosis*

Doxorubicin is one of the most effective and widely used antitumor anthracycline antibiotics. The main mechanisms of the antitumor effect of doxorubicin are associated with induction of apoptosis and inhibition of proliferative processes in tumor cells as a result of DNA degradation [2]. However, some tumors develop resistance to this drug, which is an important problem in clinical practice. Elucidation of the mechanisms involved in the regulation of tumor cell proliferation and apoptosis is important for better understanding of the causes of drug resistance.

Drug resistance of tumors can depend on intracellular inductors and inhibitors of apoptosis and bioactive substances modulating cell proliferation, *e.g.* NO. The role of this compound in the regulation of cell proliferation and apoptosis is now extensively studied [5,8].

We studied the effect of NO on the tumoritoxic effect of doxorubicin, which was evaluated by changes in DNA synthesis and tumor cell apoptosis during treatment with this drug.

MATERIALS AND METHODS

Experiments were carried out on C57Bl/6J mice (18-22 g). Ehrlich ascitic carcinoma was transplanted intraperitoneally. Ascitic fluid containing tumor cells was collected on day 7 after tumor transplantation. The cells were washed 3 times in RPMI-1640 (ICN) and adjusted to a final concentration of 10^6 cells/ml. Cell viability was evaluated by trypan blue exclusion test. Sodium nitroprusside (SNP) and sodium nitrite ($NaNO_2$, Sigma) releasing NO after cyclic reactions [3] served as NO donors. L-Arginine (chemically pure grade, manufactured in Russia) was used for endogenous generation of NO in tumor cells.

The synthesis of DNA in tumor cells was evaluated by incorporation of labeled precursor ^{3}H-thymidine (Izotop) into DNA. The cells were cultured in 96-well round-bottom plates in 200 μl culture medium containing RPMI-1640 with 10% fetal calf serum (Flow), 10 ml HEPES (Flow), 5 mM glutamine (ICN), and 40 μg/ml gentamicin in the presence of NO-generating compounds and doxorubicin in different concentrations. ^{3}H-Thymidine (0.1 μCi) was added to all wells. The plates were incubated for 18 h at 37°C and 5% CO_2. After incubation the cells were transferred to fiberglass filters using a 12-channel cell harvester (Flow). The wells were washed with normal saline (200 ml/row), cold 5% trichloroacetic acid (200 ml/row), and 96% ethanol (200 ml/row). Radioactivity of dry filters was measured in a liquid scintillation counter Mark-III (Tracor Analytic) in a Lumax scintillator (Lumac Systems Inc.). Changes in the rate of DNA synthesis were expressed in percent of the control.

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Induction of apoptosis was evaluated by the degree of chromatin degradation in cells as described previously [6]. Tumor cells were incubated with ^{3}H-thymidine (1 μCi/ml) for 24 h in the same culture medium. After label incorporation the cells were washed 3 times with RPMI-1640 and incubated for 24 h, after which apoptosis inductors were added. The content of chromatin fragments was measured after 4-h incubation with apoptosis inductors: the cell suspension was transferred into ice bath and lyzed with cold buffer (10 mM EDTA, 10 mM Tris-HCl, 0.5% Triton X-100, pH 8.0) for 15 min. The lysate was centrifuged (12,000 rpm, 10 min) and 1 ml supernatant was taken for radioactivity measurements (A_{lt}). Radioactivity of the precipitate was measured after extraction with a mixture of 1% sodium dodecylsulfate and 4 mM EDTA in 0.5 ml (A_2). The degree of chromatin degradation was estimated as the ratio of the content of chromatin fragments in the supernatant to the total label content:$(1.5\times(A_{lt}- A_{lo})/(A_2+A_{1t}-1.5A_{1o}))\times100\%$, where A_{lo} is A_{lt} value before apoptosis induction in the control.

The results were statistically processed using Student's *t* test.

RESULTS

Incubation of Ehrlich carcinoma cells with doxorubicin revealed a dose-dependent effect of the drug on DNA synthesis. Doxorubicin in concentrations $\geq10^{-6}$ M sharply inhibited ^{3}H-thymidine incorporation into DNA. Decreasing doxorubicin concentration to 10^{-7} M led to activation of DNA synthesis, which we attribute to stimulation of proliferative processes (Table 1).

Combined use of NO-generating compounds and doxorubicin significantly increased DNA synthesis in tumor cells. ^{3}H-Thymidine incorporation into DNA in cells incubated with NO donors was higher compared to cells incubated with doxorubicin in different concentrations and surpassed the control (Table 1).

Evaluation of apoptotic death by the degree of chromatin degradation also showed that this process depended on the dose of doxorubicin. Increasing antibiotic concentration led to apoptosis stimulation (Table 2). NO-generating compounds added to the incubation medium appreciably inhibited apoptosis of Ehrlich carcinoma cell induced by doxorubicin. SNP and L-arginine exhibited the highest protective effect.

Hence, the tumoritoxic effect of doxorubicin is realized through inhibition of proliferative processes and stimulation of apoptosis. NO donors appreciably reduced the antiproliferative effect of doxorubicin and apoptosis induction in tumor cells. The effects of NO-generating compounds did not depend on the mode of NO formation in cells. The effects of substances chemically generating NO (SNP and $NaNO_2$) were similar to that of L-arginine (NO-synthase reaction substratum).

TABLE 1. Effect of Doxorubicin and NO-Generating Compounds on DNA Synthesis in Ehrlich Carcinoma Cells (*M*±*m*, % of Control)

Compound	Control	Doxorubicin		
		10^{-5} M	10^{-6} M	10^{-7} M
—	100	47.82±6.70	73.0±7.5	128.0±11.2
$NaNO_2$, 10^{-5} M	137.3±11.7	184.5±8.9*	135.1±8.9*	144.6±11.7*
SNP, 10^{-5} M	153.6±10.5	134.0±12.3*	181.0±11.4*	171.3±12.3*
L-Arginine, 5 mM	131.6±9.3	84.0±7.9*	146.0±9.7*	160.7±9.8*

Note. *$p<0.01$ compared to the control.

TABLE 2. Effect of Doxorubicin and NO-Generating Compounds on Chromatin Degradation (%) in Ehrlich Carcinoma Cells (*M*±*m*, % of Control)

Compound	Control	Doxorubicin		
		10^{-5} M	10^{-6} M	10^{-7} M
—	4.2	10.7±1.6*	6.3±0.7	5.2±1.2
$NaNO_2$, 10^{-5} M	2.6±0.3	4.8±0.7**	3.5±0.4**	5.4±2.1
SNP, 10^{-5} M	2.4±0.5	2.9±0.2**	1.9±0.3**	4.5±1.9
L-Arginine, 5 mM	4.6±1.2	2.7±0.3**	1.8±0.2**	3.3±1.2

Note. $p<0.01$ compared to *control, **doxorubicin in corresponding concentrations.

The mechanism of tumor cell protection from doxorubicin damage is little studied. The leading role in its realization can belong to the catalytic unit of DNA-dependent protein kinase, a key enzyme of DNA reparation. Exposure of cells to NO increased expression of DNA-dependent protein kinase [7]. It is known that NO-mediated activation of DNA-dependent protein kinase protects cells from different DNA-damaging factors widely used in clinical practice, from radio- and chemotherapy with anthracycline antibiotics and cisplatin. Doxorubicin stimulates NO synthesis in murine mammary carcinoma cells *in vivo* [4], which is probably associated with the defense reaction of tumor cells. The authors showed that NO synthesis in cells did not potentiate therapeutic activity of doxorubicin.

Hence, NO acts as a factor protecting tumor cell DNA from damage induced by doxorubicin and can modulate tumor resistance to anthracycline antibiotics. Since some drugs generate NO [1], it is important to investigate the antitumor activity of drug combinations used in the therapy of cancer.

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