ONCOLOGY

Apoptotic Death of Human Lympholeukemia HL-60 Cells Resultant from Combined Effect of Cobalt Octa-4,5-Carboxyphthalocyanine Propylenglycol Ether and Ascorbate

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 140, No. 12, pp. 684-687, December, 2005
Original article submitted March 24, 2005

Cobalt octa-4,5-carboxyphthalocyanine propylenglycol ether proposed for antitumor therapy potentiates the cytotoxic effect of ascorbate on HL-60 human leukemia cells. Combination of these substances caused the formation of H_2O_2 in the medium and initiated apoptotic death of cells. Catalase abolished the cytotoxic effect of this combination. The results indicate that binary catalytic system of this combination can be regarded as a potential antitumor agent.

Key Words: *tumor cells; cobalt phthalocyanines; ascorbic acid; combined effect; apoptosis*

New antitumor drugs based on binary catalytic systems (BCS) including cobalt organocomplexes and ascorbic acid [1-5,7,8] are introduced in clinical practice. The cytotoxic effect of BCS is determined by their capacity to generate ROS [3]. Combination of sodium ascorbate (Asc) with cobalt octa-4,5-carboxyphthalocyanine propylenglycol ether (ETp) included in the complex with β-cyclodextrine propylenglycol ether was proposed as one of such systems. The antitumor effect of ETp+Asc combination was proved [8]. The aim of this study was to clear out whether ETp+Asc combination caused apoptotic death of tumor cells, which is an important condition for using this BSC as the antitumor drug.

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MATERIALS AND METHODS

Human lympholeukemia HL-60 cells (All-Russian Collection of Cell Cultures, Institute of Cytology, Russian Academy of Sciences) were cultured in RPMI-1640 (Sigma) with 10% FCS (HyClone), 80 μg/ml gentamicin (Gibco) at 37°C in Corning culture flasks.

For evaluation of the cytotoxic effect of ETp and Asc, the cells were seeded into culture flasks or 96-well plates (Nunc) in a concentration of 5×10^4 cell/ml. Twenty-four hours after seeding ETp and Asc (Sigma) were added at an ETp to Asc molar ratio of 1:20. The cytotoxic effect of the ETp+Asc combination was evaluated after 2 days by the number of viable cells in comparison with the control (no ETp and Asc). The kinetics of cell death was evaluated using a hemocytometer at certain time intervals after addition of the substances. Cell death was evaluated by Trypan Blue staining.

The generation of H_2O_2 by the ETp+Asc combination in culture medium was evaluated by the polarographic method, based on the increase of oxygen concentration in a medium with H_2O_2 after addition of catalase [9].

For evaluation of apoptotic cell death under the effect of the test substances by aberrant distribution of chromatin [10,13], the cells were cultured on slides or in plastic dishes. Before the analysis Hoechst 33342 (H 33342) stain (penetrating into viable cells) and ethidium bromide (penetrating into dead cells only) were added to the culture (1 μg/ml each). Live cells fluoresced green (H 33342), dead cells were orange or yellow (H33342+ethidium bromide). Cells with aberrant chromatin distribution (nucleus fragmentation, pyknosis, marginal distribution of chromatin) were considered as apoptotic [6]. This method differentiates cells at different stages of apoptosis before and after disruption of the plasma membrane.

The development of apoptosis after addition of ETp+Asc was evaluated also by flow cytometry by accumulation of cells in the sub-G_1 area [13]. Analysis of DNA cytograms was carried out on a PARTEC III flow cytometer.

Oligonucleosome fragmentation was evaluated by DNA electrophoresis in agarose gel (1.8%) [5]. For the analysis of large-scale DNA fragmentation, the cells were embedded into low-melting T7 agarose (Sigma) [14]. Agarose block containing about 150,000 cells was impregnated into a well of 1% 1-B agarose gel (Sigma) and pulse electrophoresis was carried out [15]. The distribution of DNA fragments in the gels was measured on a scanning fluorometer [4].

All experiments were repeated at least 3 times; the arithmetic means and square deviations were estimated.

RESULTS

Addition of ETp in nontoxic concentrations significantly potentiated the cytotoxic effect of Asc (Fig. 1). For instance, addition of 5 μM ETp and 100 μM Asc to cells caused a cytotoxic effect, while ETp or Asc in the same concentrations alone were nontoxic for HL-60 cells, which attests to synergic effect of the system components. ETp+Asc in the nutrient medium without cells reduced oxygen concentration and accumulation of H_2O_2 to 110 μM during the first 15 min, during the next 45 min the level H_2O_2 decreased to 50 μM. The concentration of H_2O_2 in a medium containing ETp or Asc did not surpass 10 μM. Similar results were obtained in cell culture. Addition of catalase (200 U/ml) together

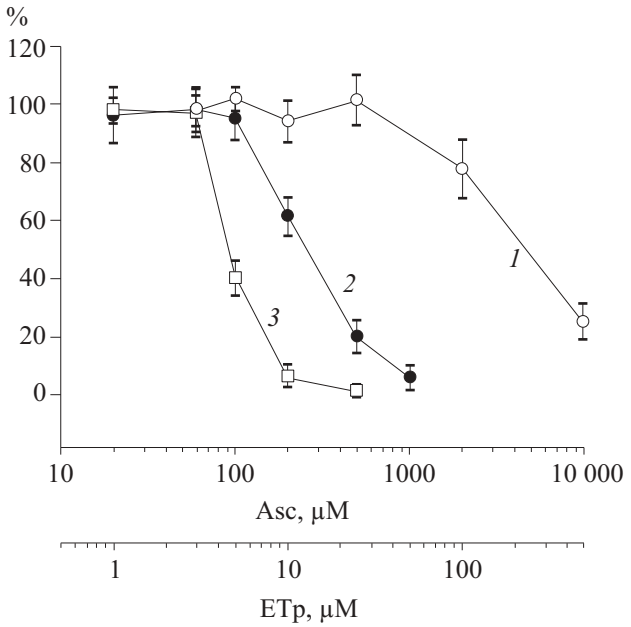

Fig. 1. Cytotoxic effect of ETp and ascorbate (Asc) on HL-60 lympholeukemia cells. Ordinate: percentage of viable cells in experimental cultures (*vs.* control) 2 days after addition of ETp (*1*), Asc (*2*), ETp+Asc in 1:20 molar ratio (*3*).

with ETp (10 μM) and Asc (200 μM) completely prevented accumulation of H_2O_2 in the medium and abolished the cytotoxic effect of ETp+Asc. Two days after addition of the substances the percentage of live cells in the experimental groups in comparison with the control was 8±3% (ETp+Asc), 95±7% (catalase+ETp+Asc), 101±8% (catalase), 6±2% (SOD+ETp+Asc), and 93±8% (SOD). SOD (up to 1000 U/ml) did not prevent H_2O_2 accumulation in the me-

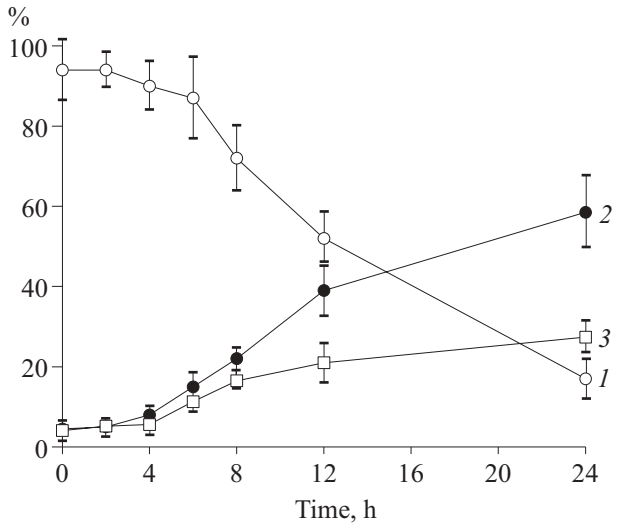

Fig. 2. Kinetics of cell death (*1*) and increase in the number of apoptotic cells (*2*, *3*) after addition of 10 μM ETp+200 μM Asc. Percentage of apoptotic cells was evaluated by aberrant distribution of chromatin (*2*) or by DNA loss by cells (percentage of sub-G_1 cells; *3*).

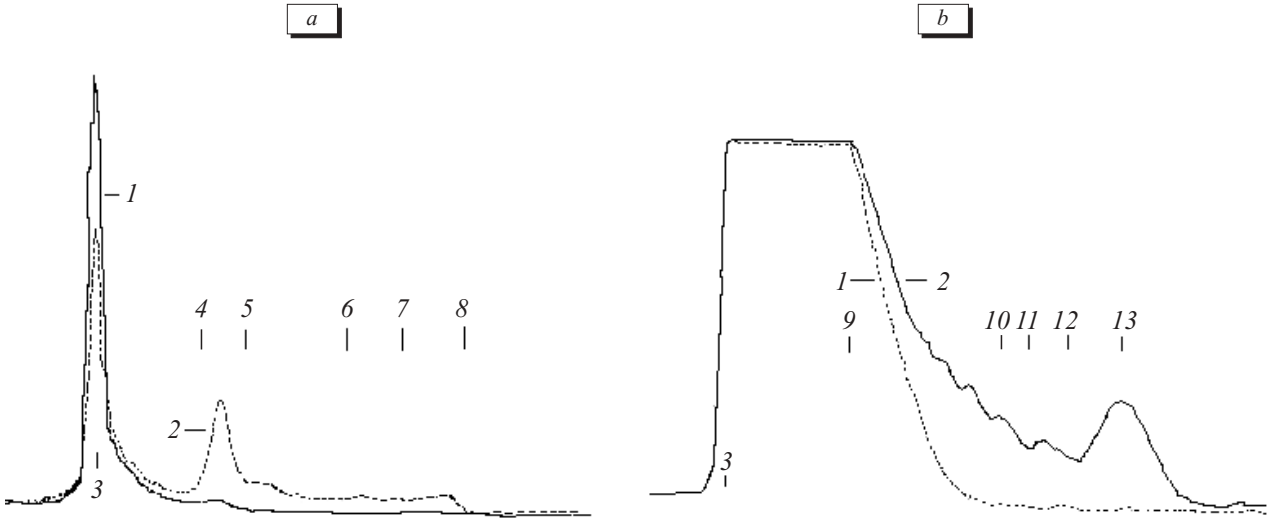

Fig. 3. Large-scale (*a*) and internucleosomal (*b*) fragmentation of DNA after addition of 10 μM ETp+200 μM Asc. Electrophoregrams of HL-60 cell DNA in 1% (*a*) and 1.8% (*b*) agarose. *1*) control cells; *2*) cells incubated for 5 h (*a*) and 14 h (*b*) with ETp+Asc; *3*) position of well center for placing the sample; *4-7*) pulse markers *Saccharomyces Cerevisiae* chromosomes: 2,200,000, 1,100,000, 555,000, 295,000 b. p.; *8*) phage *Lambda* DNA (48,500 n. p.); *9-13*) phage *Lambda Hind* III DNA restrict (2322 n. p.) and pBR 322 markers (587, 434, 267, and 184 n. p., respectively).

dium and cytotoxicity of ETp+Asc, that is, the cytotoxic effect of ETp+Asc BCS was due to generation of H_2O_2.

We studied the kinetics of cell death and kinetics of increment in the percentage of cells dead by apoptosis by morphological sign (aberrant distribution of chromatin; Fig. 2, *a*) [6,13]. The decrease in the percentage of live cells was observed 6-8 h after addition of 10 μM ETp and 200 μM Asc. During the same period the number of cells dead by apoptosis increased. Hence, according to this criterion, ETp+Asc initiated apoptotic death of cultured HL-60 human lympholeukemia cells. Flow cytometry showed similar results. Four hours after addition of ETp and Asc the percentage of cells in the sub-G_1 area of DNA cytogram increased (Fig. 2, *b*). The appearance of these cells indicates partial loss of chromatin. We observed an increase in the percentage of sub-G_1 cells during the period, when the percentage of live cells remained the same as in the control (6 h after addition of BCS, Fig. 2, *b*). Hence, after addition of ETp+Asc the cells started loosing chromatin before impairment of the plasma membrane integrity, which is a characteristic sign of apoptosis [13]. The loss of chromatin during the development of apoptotic death of cells is associated with blabbing [10], and after addition of BCS we observed an increase (*vs.* the control) in the percentage of cells containing apoptotic bodies on their surface.

During the first hours after addition of ETp and Asc, before the appearance of dead cells, we detected (by pulsed electrophoresis) large-scale fragmentation of DNA with fragments of 300,000 to 2,200,000 b. p., with a pronounced peak between 1,100,000 and 2,200,000 b. p. (Fig. 3, *a*). This fragmentation caused by double-strand DNA breaks of rosettes and coils [11] precedes internucleosomal DNA fragmentation [12], which was detected at the late stages of cell death (Fig. 3, *b*).

Hence, ETp significantly potentiated the cytotoxic effect of Asc on tumor cells. ETp+Asc BCS caused accumulation of H_2O_2 in the medium. Catalase abolished the cytotoxic effect of ETp+Asc. ETp+Asc induces the formation of double-strand DNA breaks in tumor cells causing large-scale fragmentation of DNA at the early stages of the action of the binary system and subsequent internucleosomal fragmentation. Impairment of chromatin structure and loss of DNA by cells as a result of ETp+Asc effect precedes impairment of the plasma membrane integrity and cell death. Hence, ETp+Asc initiates apoptotic death of tumor cells and can be regarded as a potential antitumor agent.

The authors are grateful to Prof. R. I. Yakubovskaya and Prof. Yu. V. Evtodienko for support and fruitful discussion. The study was carried out within the framework of Moscow Program for Oncology.

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