BIOPHYSICS AND BIOCHEMISTRY

Generators of Reactive Oxygen Forms γ-Irradiation and Ascorbic Acid—Cobalt Metallocomplexes Induced Large-Scale Fragmentation and Reparation of DNA in Tumor Cells

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It was demonstrated that ascorbate—cobalt phthalocyanine complex produces a time-dependent nuclease effect on leukemia K-562 cells is. Catalase added to the incubation medium prevented or blocked fragmentation of cell DNA. The size of large-scale fragments formed during irradiation and exposure to the above system varied from 2200 to 30 kbp. The fragments induced by the system recombined slower than the fragments induced by γ-irradiation in a dose adequate by the level of DNA damage. This effect observed previously in HEp-2 carcinoma cells exposed to the action of the B_{12b}+C vitamin system can be explained by generation of H_2O_2 inducing more severe damage to DNA structure than γ-radiation due to site-specific Fenton reaction.

Key Words: *ascorbic acid; cobalt phthalocyanine; vitamin B_{12b}; large-scale DNA fragments; tumor cells*

The study of the mechanism of the genotoxic effect of cobalt metallocomplexes with corrin (vitamin B_{12b}) and phthalocyanine (teraphthal, TP) ligands in combination with ascorbic acid (AA) on tumor cells is dictated by the fact that binary catalytic systems are proposed for clinical practice as new antitumor drugs [2,7]. For instance, TP+AA system is now tested in Research Institute of Clinical Oncology (Russian Cancer Research Center).

It was found that the AA+TP system produces a cytotoxic effect on tumor cells *in vitro* and on tumors *in vivo* [6,7], generates (similarly to the B_{12b}+C vitamin system [4]) reactive oxygen species (ROS), induces DNA and RNA breaks in solutions [2,6] and large-scale and oligonucleosome fragmentation of DNA in tumor cells *in vitro* [1,5].

Here we studied the formation of large-scale DNA fragments, early apoptosis markers, in leukemia K-562 cells under the action of two ROS generators, γ-irradiation and binary TP+AA system. Moreover, it was interesting to find out whether the AA—cobalt phthalocyanine system can induce the formation of poorly recombining DNA fragments (compared to γ-irradiation).

MATERIALS AND METHODS

Human myeloid leukemia cells K-562 were obtained from All-Russian Collection of Cell Cultures

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(Institute of Cytology, Russian Academy of Sciences, St. Petersburg). The cells were grown in RPMI-1640 medium (Sigma) containing 40 μg/ml gentamicin and 10% FCS (Flow) at 37°C in atmosphere containing 5% CO_2. For evaluation of the genotoxic effect of TP we used a combination of AA with cobalt octa-4,5-carboxyphthalocyanine propylene ether introduced into complex with cyclodextrin (Research Institute of Organic Preproducts and Paints). The cells (50,000 per 1 ml medium) were seeded to culture flasks; TP and AA were added 1 day after seeding in a molar ratio of 1:20. TP and AA added individually had no cytotoxic effect, which agreed with the concept of binary theory. Catalase was added as described previously [1], the cells were γ-irradiated on a GUBE setup (Cs^{137}, 2.4 Gy/min dose power).

During the analysis of DNA fragmentation, the cells were embedded into low-melting agarose T7 (Sigma). Lysis and enzyme digestion of cells embedded into agarose [11] prevented fragmentation of large-sale DNA observed during cell lysis in suspension. The agarose block containing about 150,000 cells was transferred into a cell in 0.25% agarose 1-B gel (Sigma) and electrophoresis was carried out for 21 h at a voltage of 0.7 V/cm and gel length of 14 cm. Otherwise, the agarose block was placed into a cell of 1% agarose 1-B gel and pulse electrophoresis was carried out as described previously [1]. The electrophoregrams consisted of two fractions. The fraction of high-molecular weight DNA remained in the start cell, while the fraction of large-scale DNA (≥2200 kbp [1]) migrated from it. The degree of DNA degradation was measured on a scanning fluorometer [4], the percent of fragmented DNA from the total DNA content in the row was calculated. The use of continuous electrophoresis in a gel with low agarose content and pulse electrophoresis in 1% agarose determined close results for measurements of the percent of fragmented DNA.

Experiments were repeated at least 3 times, the data were processed statistically using Student's *t* test.

RESULTS

Our previous studies showed that the yield of DNA fragments in K-562 cells depended on the concentration of components of the cobalt phthalocyanine-AA system [5]. The nuclease effect of the TP+AA system depended also on the time (the content of fragmented DNA increased from 20 to 45% with increasing the incubation time from 1 to 4 h). The spectrum (Fig. 1) of large-scale DNA fragments formed under these conditions ranged from

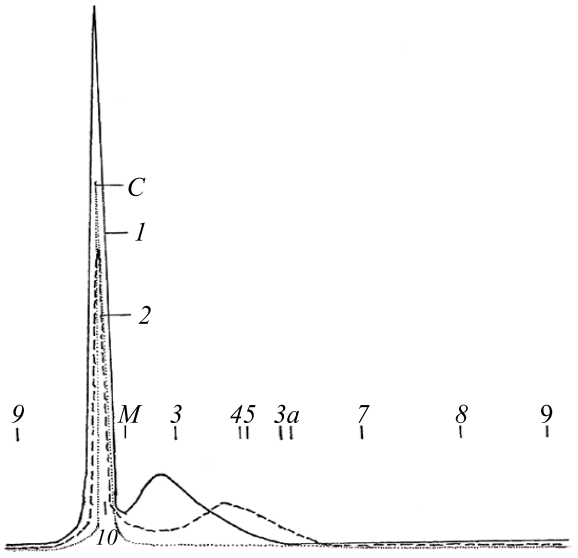

Fig. 1. Electrophoregram of DNA from K-562 cells in 0.25% agarose gel. Abscissa: distribution of DNA fragments in 14-cm gel. *C*: control cells; *1*) cells incubated with 5 μM TP+100 μM AA; *2*) cells irradiated in a dose of 35 Gy at 4°C; *M*: *S. cerevisiae* marker 2200 kbp; *3*) 1000 kbp marker; *3a*) 50 kbp marker; *4*) T4 phage DNA, 168 kbp; *5*) T5 phage DNA, 103 kbp; *6*) phage λ DNA, 48.5 kbp; *7-8*) phage λ Hind III restriction fragments, 23.1 and 9.4 kbp; *9*) gel start and stop marks; *10*) center of the cell for the sample. The location of 220-225-kbp pulse markers are presented in article [1]. Ordinate: fluorescence intensity (rel. units).

2200 to 50 kb with a pronounced maximum at 1000 kbp, which was determined by degradation of more high-molecular weight chromatin structures (DNA turns and rosettes) [9]. γ-Irradiation in the applied doses led to deeper DNA fragmentation than the binary system and induced the appearance of small quantities of fragments <50 kbp, which attested to possible cleavage of DNA loops [9]. Oligonucleosome DNA fragments were not formed, because leukemia K-562 cells are resistant to the majority of physical and chemical genotoxins by this apoptosis sign [12].

It was found that DNA fragments formed under the effect of chemical nuclease (combination of B_{12b} and C vitamins) on HEp-2 laryngeal epidermoid carcinoma cells recombined slower than the fragments formed after γ-irradiation in the dose adequate by the level of DNA damage [4].

γ-Irradiation of K-562 leukemia cells in the absence of DNA reparation (at 4°C) induced fragmentation, which increased with increasing the irradiation dose and attained 35% at a dose of 20 Gy. This is close to the nuclease effect of 2 h exposure with 5μM TP+100 μM AA (Fig. 2). After irradiation in the same dose 20 Gy but at 37°C, the induced DNA breaks were almost completely repaired over 1 h and the irradiated cells did not differ from the control by the fragmentation level (Fig. 2).

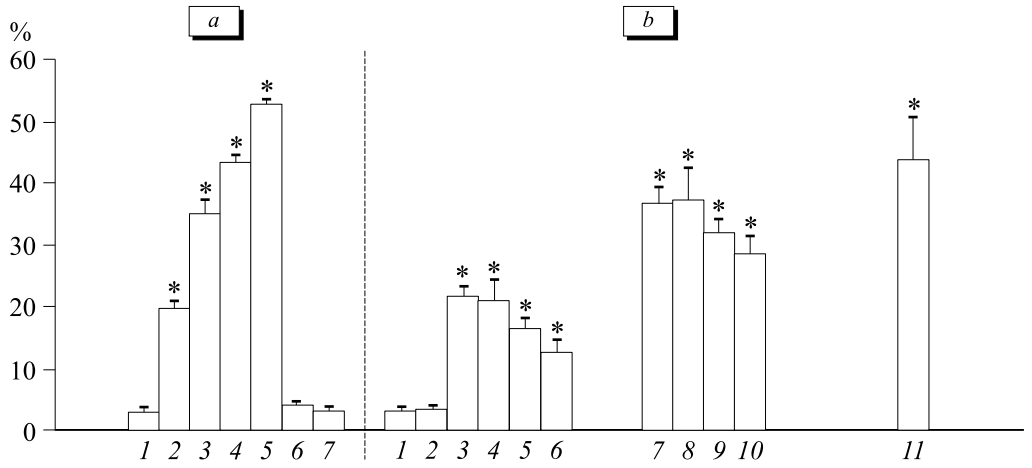

Fig. 2. Content of fragmented DNA in K-562 cells depending on the conditions of g-irradiation (*a*), duration of incubation with 5 μM TP+100 μM AA, and duration of incubation after addition of catalase (*b*). Fragment *a*: *1*: control cells; *2-5*: irradiation in doses of 10, 20, 35, and 50 Gy at 4°C; *6-7*: irradiation in a dose of 20 Gy at 37°C, incubation for 1 and 2 h from the start of irradiation, respectively. Fragment *b*: *1*: control cells; *2*: cells incubated with 5 μM TP+100 μM AA, addition of catalase with TP+AA; *3-6*) incubation with TP+AA for 1 h (*3*), addition of catalase and incubation for 1 h (*4*), 2 h (*5*), and 4 h (*6*); *7-10*) incubation with TP+AA for 2 h (*7*), addition of catalase and incubation for 1 h (*8*), 2 h (*9*), and 4 h (*10*); *11*) incubation with TP+AA for 4 h. **p*<0.05 compared to control cells. Ordinate: migration of fragmented DNA from the cell with the sample.

In the study of reparability of large-scale DNA fragments induced by the test system we did not wash the exposed cells from the components (as in previous experiment [4]), but added catalase to the incubation medium after certain time intervals, which prevented the cytotoxic effect of AA both in combination with metals of alternating valency and during auto- and catalytic oxidation of AA [1,6, 14,15]. In preliminary experiments we showed that addition of catalase to the incubation medium completely prevented not only the cytotoxic effect of AA [1,14], but also large-scale DNA fragmentation (Fig. 2). After 1-h incubation of K-562 cells with the binary system, addition of catalase, and subsequent recovery for 1 h, the content of DNA fragments did not change (Fig. 2), and after 2 and 4-h reparation, the content of fragments decreased by 25 and 50%, respectively (Fig. 2). Similar by its dynamics, but less pronounced reparation was observed in K-562 cells after more severe DNA damage caused by 2-h incubation with TP+AA (Fig. 2): about 80% formed fragments did not recombine after 4-h reparation. These findings suggest that both chemical nucleases (B_{12b}+C vitamins [4] and the test AA+TP system) produced comparable damaging effects on DNA of tumor cells and induced the formation of slowly recombining DNA fragments.

Decelerated recombination of DNA fragments formed in K-562 and HEp-2 cells incubated with the AA-cobalt metallocomplex system (compared to that in cells exposed to γ-radiation) can be explained by the formation of H_2O_2 in the binary systems, which induced more severe damage to DNA structure due to site-specific Fenton reaction [15]. Local damage to DNA is probably promoted by activation of endonucleases [8,13] initiated by disturbances in intracellular Ca compartmentalization [13,14] observed during cell incubation with AA and H_2O_2 in toxic concentrations, but absent after X-ray exposure [3].

Hence, the cytotoxic effect of the binary system and H_2O_2 produced by these systems is not restricted by large-scale DNA degradation observed by us and other investigators [1,4,8,10]. It is known that H_2O_2 also damages other chromatin components, induces protein denaturation and LPO, violates the integrity of nuclear membranes, which in turn also initiates the formation of large DNA fragments [10] and, probably, impairs recombination of DNA fragments appearing due to other mechanisms. Thus, large-scale DNA fragmentation should be considered not only as the early apoptosis marker [5, 10], but also as an integral indicator of structuredness and integrity of chromatin in cells subjected to the damaging factors of different nature and acting via different mechanisms.

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