Combined Treatment with Vitamin B_{12b} and Ascorbic Acid Causes *in Vitro* DNA Degradation in Tumor Cells

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Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 131, No. 4, pp. 434-436, April, 2001
Original article submitted November 16, 2000

Incubation of Ehrlich ascites carcinoma and HEp-2 human epidermoid laryngeal carcinoma cells with hydroxycobalamin (vitamin B_{12b}) and ascorbic acid induced generation and accumulation of double-stranded DNA fragments (23,000 b.p. and longer) in cells. The same vitamins alone in the same concentrations produced no such effects. DNA degradation in HEp-2 cells caused by long-term (4 h) incubation with 5-25 μM hydroxycobalamin and ascorbic acid (1:10-1:40 molar ratio) at 37°C was comparable with that induced by γ-irradiation in a dose of 150 Gy at 4°C.

Key Words: *vitamin B_{12b}; hydroxycobalamin; ascorbic acid; DNA degradation; tumor cells*

The search for new antitumor preparations is a topical problem of biology and medicine. Growing interest to the mechanisms underlying combined action of cobalt-containing complexes and ascorbic acid (AA) on tumor cells is stimulated by the fact that these binary catalytic complexes (*e.g.*, vitamin B_{12b} and ascorbate) were proposed, tested, and patented as antitumor agents [1,3,6]. The effects of these catalytic systems are associated with generation of reactive oxygen species $O_2^{\bullet-}$, H_2O_2, and $OH^{\bullet}$ [1-3,5]. Previous studies showed that cobalt-containing corrin complexes, in particular, vitamin B_{12b}, in combination with AA exhibit *in vitro* nuclease activity and induce DNA breaks in pBR 322 plasmid [2]. A question arises: whether vitamin B_{12b} and AA cause DNA degradation in tumor cells.

Here we studied the effect of combined treatment with vitamin B_{12b} and AA on *in vitro* DNA degradation in Ehrlich ascites carcinoma (EAC) and HEp-2 human epidermoid larynx carcinoma cells.

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MATERIALS AND METHODS

EAC cells grown in the peritoneal cavity of outbred mice for 7 days were washed with Hank's solution and suspended in RPMI-1640 medium (Sigma) containing 10% bovine serum, 10 mM HEPES, and 80 μg/ml gentamicin. Hydroxycobalamin (vitamin B_{12b}, Kurgan Plant of Medicinal Preparations) and AA (Sigma) were added immediately after resuspension. HEp-2 cells were grown in Dulbecco's modified Eagle medium (Sigma) containing 10% fetal bovine serum (Sigma) and 80 μg/ml gentamicin at 37°C and 5% CO_2. Vitamin B_{12b} and AA were added to cell culture 1 day after inoculation in a concentration of 5×10^4/ml.

Vitamin B_{12b} and AA were dissolved in Hank's solution immediately before use. The cells were incubated with vitamin B_{12b} and AA in the dark at 37°C. Cells in the suspension were counted in a hemocytometer. Cell viability was evaluated by trypan blue exclusion test. After incubation EAC cells were washed 3 times by centrifugation in ice-cold Dulbecco's phosphate buffer saline at 600*g* for 2 min. HEp-2 cells were harvested from plastic flasks with a silicone scraper after removal of medium containing serum, vitamin B_{12b}, and AA and washed by centrifugation. Some

0007-4888/04/1314-0364$25.00

HEp-2 cells in a serum-containing medium were γ-irradiated (^{137}Cs, 150 Gy) using a GUBE device at 4°C.

Fragmentation of double-stranded DNA was assayed by the method [7] with modifications. Aliquots of cell suspension (3-5×10^5 cells) were centrifuged, cell lysis and degradation of proteins and RNA were performed in a medium containing 10 mM EDTA, 50 mM Tris-HCl buffer (pH 8.0), 0.5% sodium lauryl sarcosinate, 0.5 mg/ml protein kinase K, and 0.5 mg/ml RNase A (Sigma) at 50°C for 15-22 h. Electrophoresis in 0.75% agarose gel was performed in Tris-borate buffer (pH 8.0) containing 0.5 μg/ml ethidium bromide for 4 h (1.2 V/cm gradient).

DNA photofluorograms were similar to those obtained after electrophoresis of lysed control and γ-irradiated mammalian cells [8,9] and consisted of 2 fractions: high-molecular weight DNA (HMW-DNA) remained at the start point and fragmented DNA was characterized by lower electrophoretic mobility than lambda phage DNA restrict containing 23,000 b.p. and used as the marker. The gels were scanned on a fluorometer-densitometer [4] and DNA degradation was estimated by the content of HMW-DNA (percentage of total DNA content in gel). The results were analyzed by Student's *t* test.

RESULTS

Vitamin B_{12b} (5 and 100 μM) and AA (50 and 200 μM) alone had no nuclease activity. The content of HMW-DNA in cells incubated with 5 and 100 μM vitamin B_{12b} (85.1±30 and 88.2±2.0%, respectively) and 50 and 200 μM AA (83.3±1.0 and 86.8±2.2%, respectively) did not differ from the control (85.8± 1.0%). Incubation of EAC cells with vitamin B_{12b} and AA in concentrations of 5 and 50 μM, respectively, led to time-dependent DNA degradation (40% HMW-DNA after 4 h, Fig. 1). The effect was more pronounced after increasing the concentration of AA in the incubation medium to 200 μM. The contents of HMW-DNA were 40 and 10% cell DNA after incubation with vitamin B_{12b} and AA for 1 and 4 h, respectively.

Similar results were obtained in experiments with monolayer cultures of HEp-2 human epidermoid larynx carcinoma cells. Vitamin B_{12b} and AA alone did not induce DNA fragmentation: the contents of HMW-DNA after 2-h incubation with vitamin B_{12b} and AA were 84.0±4.5 and 77.0±1.5%, respectively (*vs.* 84.7± 3.0% in the control). Combined treatment with vitamin B_{12b} and AA in concentrations of 25 and 500 μM, respectively, produced a time-dependent decrease in the content of HMW-DNA in HEp-2 cells (55.2±2.3 and 24.8±2.2% after 1- and 2-h incubation, respectively, $p<0.05$ compared to the control). The effect of 2-h incubation of HEp-2 cells with vitamin B_{12b} and AA

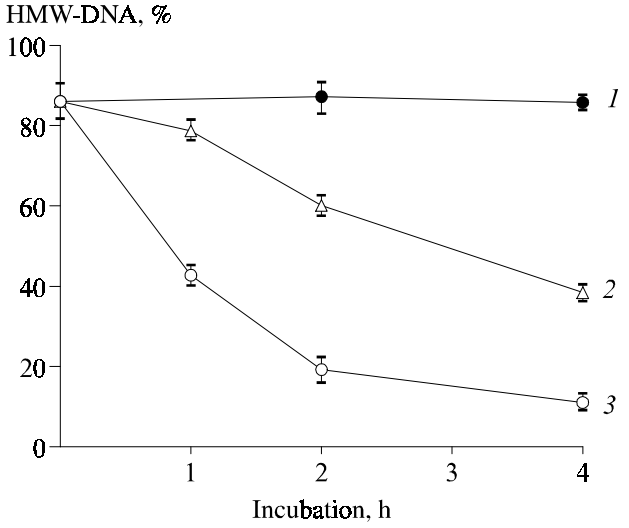

Fig. 1. Content of high-molecular-weight DNA (HMW-DNA) in Ehrlich ascites carcinoma cells in the control (*1*) and after combined treatment with 5 μM vitamin B_{12b} and 50 (*2*) or 200 μM (*3*) ascorbic acid (AA). *$p<0.05$ compared to the control.

was similar to that observed after 2-h incubation of EAC cells with these agents in much lower concentrations (5 and 200 μM, respectively).

We compared the degree of DNA degradation caused by vitamin B_{12b} and AA with the nuclease effect of ionizing radiation producing OH radicals. After γ-irradiation of HEp-2 cells with 150 Gy at 4°C, the content of HMW-DNA was 31.0±1.6% in the absence of DNA reparation ($p<0.05$ compared to the control). This effect was similar to that observed after combined treatment with vitamin B_{12b} and AA in concentrations of 25 and 500 μM, respectively, at 37°C for 2 h. γ-Irradiation and incubation of EAC or HEp-2 cells with vitamin B_{12b} and AA in various concentrations led to the formation DNA fragments with electrophoretic mobility lower than that of 23,000 b.p DNA restrict. [4,9].

Thus, combined treatment of EAC and HEp-2 cells with hydroxycobalamin (5-25 μM) and AA (50-200 and 500 μM, respectively) caused DNA degradation. The same preparations alone produced no such effect. Damages to tumor cell DNA increased with increasing the duration of incubation.

Since DNA damages caused by vitamin B_{12b} and AA remain unrepaired for 4 h, they probably result in chromosomal aberrations and reproductive death of EAC and HEp-2 cells offer their enter into mitosis. The vitamin B_{12b}-AA system exhibits pronounced genotoxicity comparable with that of γ-irradiation in a dose 150 Gy, which far surpasses the lethal dose for mammalian cells. These data suggest that EAC and HEp-2 cells damaged by OH radicals do not enter mitosis and die during the interphase.

Thus, vitamin B_{12b} and AA alone did not cause death of EAC and HEp-2 cells. Combined treatment

of EAC cells with 5 μM vitamin B_{12b} and 50-200 μM AA, as well as incubation of HEp-2 cells with 25 μM vitamin B_{12b} and 500 μM AA, did not change the count of viable cells (95%). Thus, DNA degradation in EAC and HEp-2 cells under the effect of vitamin B_{12b} and AA preceded disintegration of plasma membranes and cell death. The count of viable cells markedly decreased after 24-h incubation with vitamin B_{12b} and AA. The survival rates of EAC cells incubated with 5 μM vitamin B_{12b} and 50 or 200 μM AA were 50 and 20%, respectively. After incubation of HEp-2 cells with 25 μM vitamin B_{12b} and 500 μM AA, their survival rate was 20%.

Our results indicate that the vitamin B_{12b}-AA system possesses pronounced genotoxic activity, while the vitamins alone do not have this property. DNA damages caused by these vitamins precede death of tumor cells.

Authors are grateful to Prof. Yu. V. Evtodienko for the help in this experiment and participation in discussions of the results.

This work was performed within the framework of the Moscow Program on Oncology.

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