ONCOLOGY

Oxidative Stress in HEp-2 Human Laryngeal Carcinoma Cells Induced by Combination of Vitamins B_{12b} and C

V. S. Akatov, M. E. Solov'eva, V. V. Leshchenko, and V. V. Teplova*

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 136, No. 9, pp. 318-321, September, 2003
Original article submitted December 16, 2002

Incubation of human laryngeal epidermoid carcinoma HEp-2 cells with hydroxocobalamin (vitamin B_{12b}) and ascorbic acid (vitamin C) for 1 h initiated oxidative stress accompanied by damage to mitochondria and increase in intracellular oxidative activity. Studies of the kinetics of these processes showed that the increase in intracellular H_2O_2 activity and mitochondrial damage are more likely a result, but not the cause of cell apoptosis during the first hour of their incubation with vitamins B_{12b} and C.

Key Words: *tumor cells; vitamin B_{12b}-oxycobalamine; ascorbic acid; oxidative stress; mitochondrial damage*

It was previously shown that the effect of a new antitumor drug based on a combination of vitamins B_{12b} and C (B_{12b}/C) [3] is realized via generation of AOF [6]. Irreversible damage to DNA, inhibition of mitotic activity, and triggering the death program of HEp-2, ACE, NIH-OVCAR, NS0, P388, and K562 tumor cells are observed 1 h after addition of B_{12b}/C [1,2,4-6]. Mitochondria are known to play an important role in AOF generation and antioxidant defense of cells [9]. They can modulate activities of endonucleases and induction of apoptosis through regulation of Ca^{2+} concentration. The development of oxidative stress in cells is paralleled by inhibition of functional activity of mitochondria: damage to mitochondrial DNA [9], decrease in glutathione content [8] and membrane potential of the mitochondria ($\Delta\Psi_M$), and impairment of Ca^{2+}-accumulating capacity (calcium capacity) [10]. Despite the fact that B_{12b}/C initiates cell death via generation of H_2O_2 in the medium and catalase completely inhibits cytotoxicity of this vitamin combination [6], it remains unclear whether the program of cell death is triggered by the increase in oxidative activity. We tried to clear out whether the initiation and realization of cell death program after incubation with B_{12b}/C is due to the increase in H_2O_2 activity in cells and mitochondrial damage. To this end, we investigated the state of mitochondria ($\Delta\Psi_M$ and Ca^{2+} capacities) and intracellular content of H_2O_2 in terms typical of initiation of tumor cell death under the effect of B_{12b}/C (1 h) and during further realization of the program of cell death.

Tissue Engineering Group, *Laboratory of Cell Biochemistry and Bioenergetics, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino. ***Address for correspondence:*** v-akatoff@mail.ru. Akatov V. S.

MATERIALS AND METHODS

HEp-2 cells were cultured as described previously [1,2,6]. All experiments on cell cultures were carried out during the phase of exponential growth. Inhibition of cell proliferation was evaluated by the ratio of viable cells in experimental and control cultures 48 h after addition of damaging agents. To this end the cells were detached from the bottom of culture flasks and counted using a hemocytometer. Cell viability was evaluated by Trypan blue exclusion.

Hydroxocobalamin (vitamin B_{12b}; Kurgan Pharmaceutical Plant) and ascorbic acid (vitamin C; Sigma) were dissolved in Hanks solution directly before addition to cell cultures.

For measuring calcium capacity of mitochondria, cell suspension (2×10^5 cells/ml) was incubated for 1 h with B_{12b}/C (25 μM/500 μM) in DMEM at 37°C, then washed from vitamins, suspended, and incubated in the growth medium at 37°C. Cell aliquots were collected for analysis after 0, 3, and 6 h and washed in a medium containing 150 mM NaCl, 5 mM KCl, and 10 mM Tris-HCl (pH 7.4). Measurements of Ca^{2+} capacity and $\Delta\Psi_M$ were carried out using a tetraphenylphosphonium-sensitive (TPP^+) electrode in a medium containing 100 mM KCl, 2 mM KH_2PO_4, 10 mM Tris-HCl, 5 mM succinate (pH 7.4), and 5 mM rotenone. Cell suspension (2×10^6 cells/ml) was permeabilized with 30 μM digitonine and loaded with $CaCl_2$ (12.5-μM portions) until complete inhibition of the recovery of mitochondrial membrane potential, which corresponded to complete inhibition of Ca^{2+} accumulation in mitochondria [10].

Activity of intracellular H_2O_2 was recorded using a fluorescent probe 2',7'-dichlorofluoresceine diacetate (DCFHDA) [7] at λ=530 nm on a Perkin-Elmer MPF-44B spectrofluorimeter (the fluorescence was excited at l=485 nm). Changes in H_2O_2 activity in cells were evaluated by the ratio of signals in experiment and control (K=Ie/Ik). Cell suspension (2×10^5 cells/ml) was incubated for 30 min in a medium with 20 μM DCFHDA, DCFHDA was removed from the medium by centrifugation, the cells were resuspended in phenol red-free DMEM supplemented with 10% serum and adjusted to a concentration of 10^6 cells/ml, B_{12b}/C or H_2O_2 was added, and the fluorescence kinetics was recorded for 4 h. The fluorescence kinetics of DCFHDA-loaded cells without damaging agents (control) was measured similarly.

RESULTS

In order to evaluate the role of mitochondria in B_{12b}/C-induced death of HEp-2 cells, their capacity to accumulate Ca^{2+} and maintain $\Delta\Psi_M$ after 1-h incubation with the vitamin combination B_{12b}/C (25 μM/500 μM) was studied. As was previously found, this incubation followed by removal of the vitamins B_{12b}/C from the medium was sufficient for irreversible initiation of cell death, and the number of dead cells started to increase 6-8 h after incubation [2]. The increase of TPP^+ in the medium after addition of 12 μM Ca^{2+} into suspension of digitonin-permeabilized cells attested to a drop of membrane potential, and subsequent decrease in TPP^+ attested to an increase in membrane potential and calcium accumulation in mitochondria.

The mitochondria of cells preincubated with B_{12b}/C had a higher membrane potential after 1-h incubation in comparison with control cells. This can be due to adaptation to the stress exposure. Except for these minor differences, the mitochondria of control and experimental cells virtually did not differ by the capacity to bind Ca^{2+} ions (Fig. 1, *a*).

Six hours after 1-h incubation with B_{12b}/C the state of the mitochondria in control HEp-2 cells virtually did not differ from the initial state, while Ca^{2+} capacity of mitochondria in experimental cells decreased

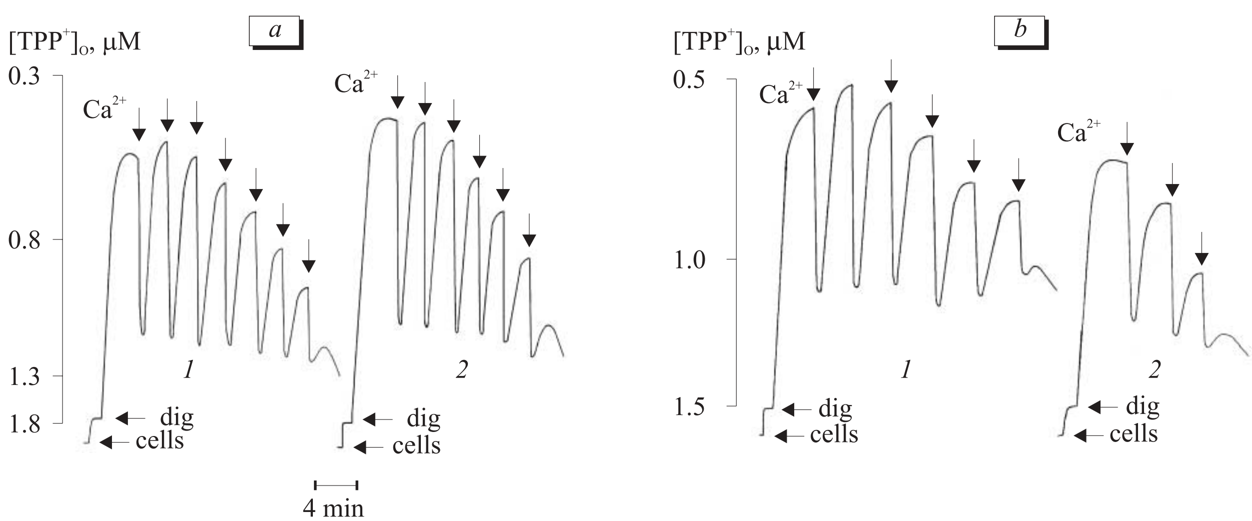

Fig. 1. Changes in mitochondrial membrane potential and calcium capacity in HEp-2 cells cultured in growth medium after 1-h incubation with vitamins B_{12b} (25 μM) and C (500 μM) (*2*) or without them (control *1*). *a*) directly after incubation; *b*) 6 h after incubation.

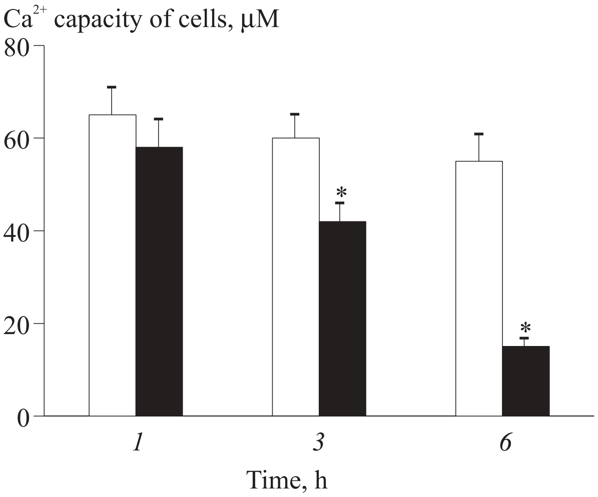

Fig. 2. Changes in calcium capacity of mitochondria in HEp-2 cells in control (light bars) and experiment (dark bars) after 1-h incubation with vitamins B_{12b} (25 μM) and C (500 μM).

3-fold and their $\Delta\Psi_M$ slightly (by 10-15%) decreased (Fig. 1, *b*).

Mitochondrial Ca^{2+} capacity of control cells virtually did not change after initiation of cell death (1-h incubation with vitamins B_{12b} and C). In experimental cultures the status of the mitochondria was at the same level as in the control 1 h after cell death initiation, and their gradual damage was observed by the third and sixth hours after B_{12b}/C treatment (Fig. 2).

Since the effect of B_{12b}/C combination is realized via generation of H_2O_2 and its toxicity is abolished in the presence of catalase (but not SOD) in the medium [6], it can be hypothesized that H_2O_2 penetrates into cells and caused injuries eventually leading to their death, or that H_2O_2 is generated in cells as a result of absorption of B_{12b}/C components. We measured the

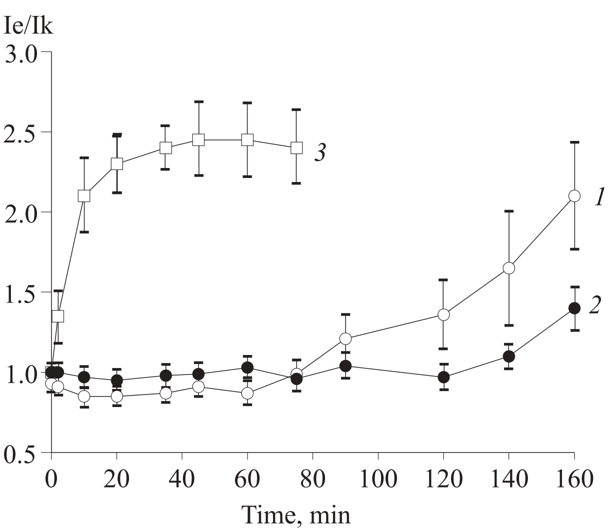

Fig. 3. Changes in H_2O_2 activity in HEp-2 cells after 1-h incubation with vitamins B_{12b} (25 μM) and C (500 μM) (*1*), 100 μM H_2O_2 (*2*), or 2 mM H_2O_2 (*3*) compared to the control.

content of H_2O_2 in cells during initiation of their irreversible damage (1-h exposure to B_{12b}/C) and during subsequent realization of the program of cell death (4 h after death initiation). The content of H_2O_2 in experimental cells was lower than in control cells during the first hour of B_{12b}/C exposure, when H_2O_2 generation in the medium was the most active [6]. This can be due to activation of the cell antioxidant system in response to AOF generated by the B_{12b}/C combination (Fig. 3). Oxidative activity in cells started to increase after initiation of their death, and 3 h after treatment with B_{12b}/C intracellular H_2O_2 activity 2-fold surpassed the control, which indicates impairment of the cellular antioxidant defense system. Addition of 100 μM H_2O_2, which inhibited cell proliferation by 50%, did not change fluorescence in experimental cell in comparison with the control during 1-2 h (Fig. 3, *2*), but after 4 h activity of H_2O_2 in cells surpassed the control level by 60%. Addition of 2 μM H_2O_2 during the first minutes caused a rapid increase in H_2O_2 activity in cells (Fig. 3, *3*). The redox system failed to control the H_2O_2 flow into the cell in this case, and the results indicate the adequacy of the method for measuring intracellular H_2O_2 using DCHFDA [8]. Cell viability during the experiments was 94-97%, characteristic of control cultures (Fig. 3). Cell death started 6-8 h after treatment with B_{12b}/C or 100 μM H_2O_2 and after 2-4-h of treatment with H_2O_2.

Hence, initiation of apoptotic cell death during the first hour of treatment with the vitamin combination B_{12b}/C is not due to impairment of mitochondrial function or increase in H_2O_2 activity in cells. Subsequent realization of the cell death program during 6 h was paralleled by mitochondrial damage and increase in H_2O_2 activity in cells, *i.e.* by the development of oxidative stress. This result is in line with previous findings, according to which the drop of intracellular glutathione started only during the first hour of B_{12b}/C action, when apoptotic cell death was triggered; glutathione concentration remained high (about 60% of the control) [1,6]. Exhaustion of glutathione pool was observed 2-3 h after triggering of the cell death program. By this time we observed mitochondrial damage and increase in H_2O_2 activity in cells. Therefore, oxidative stress in cells caused by vitamins B_{12b} and C is a result, but not the cause of initiation of the apoptosis program.

The study was supported by Moscow Program for Oncology and a grant "Universities of Russia" (No. 11.01.030).

REFERENCES

1. V. S. Akatov, Yu. V. Evtodienko, A. I. Medvedev, *et al.*, *Dokl. Akad. Nauk*, **373**, No. 6, 838-840 (2000).

2. V. S. Akatov, V. V. Leshchenko, A. A. Kudryavtsev, *et al.*, *Ros. Onkol. Zh.*, No. 6, 22-25 (2001).
3. M. E. Vol'pin, N. Yu. Krainova, I. Ya. Levitin, *et al.*, *Ros. Khim. Zh.*, **42**, No. 5, 116-127 (1988).
4. A. I. Medvedev, V. S. Akatov, Yu. V. Evtodienko, *et al.*, *Tsitologiya*, **43**, No. 3, 274-277 (2001).
5. A. I. Medvedev, V. S. Akatov, N. D. Kreshchenko, *et al.*, *Byull. Eksp. Biol. Med.*, **131**, No. 4, 434-436 (2001).
6. V. S. Akatov, Yu. V. Evtodienko, V. V. Leshchenko, *et al.*, *Biosci. Rep.*, **20**, 411-417 (2000).
7. D. M. Hockenbery, Z. Oltvai, X.-M. Yin, *et al.*, *Cell*, **75**, 241-251 (1993).
8. N. Jha, O. Jurma, G. Lalli, *et al.*, *J. Biol. Chem.*, **275**, 26,096-25,101 (2000).
9. V. P. Skulachev, *FEBS Lett.*, **492**, 1-3 (2001).
10. V. V. Teplova, A. A. Kudrjavtsev, I. I. Odinokova, *et al.*, *Biochem. Mol. Biol. Int.*, **45**, 501-510 (1998).