Modification of Intracellular Level of Free Radicals and Apoptosis in Cultured Human Endotheliocytes and Carcinoma Cells

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The intracellular levels of superoxide O_2^- and nitric oxide NO were directly measured under intravital conditions in cultured human endotheliocytes ECV 304 and carcinoma cells HeLa G-63. Comparative analysis of changes in the intracellular levels of superoxide and NO induced by ascorbic acid revealed a negative correlation between NO and O_2^- levels, whose strength depended on concentration of the acid. In pharmacological concentrations, ascorbic acid induced apoptotic death of carcinoma cells, but did not trigger apoptosis of endotheliocytes. The study demonstrated a possible way of cytotoxic action of ascorbic acid in pharmacological concentrations.

Key Words: *superoxide O_2^-; nitric oxide NO; ascorbic acid; HeLa G-63; ECV 304; cytometry*

Oxygen radicals (O_2^-, $O^\bullet$, O^{-1}) and their derivatives (OH^-, NO, NO^+, $ONOO^-$) are the key elements in the control of both physiological and pathological processes. Unfavorable environmental factors can disturb the balance of the intracellular levels of these radicals, which promotes the development of pathological processes. Ascorbic acid (AA) can demonstrate both antioxidant and prooxidant properties. Being a donor of electrons, AA acts as a cofactor for some enzymes [7]. For example, AA-induced stabilization of tetrahydrobiopterin, a cofactor of NO-synthase, leads to activation of the enzyme and elevation of intracellular NO [8]. AA exhibits also some non-enzymatic activities. It is a potent water-soluble scavenger antioxidant, which can neutralize such radicals as $O_2^{\cdot-}$, $HO_2^\bullet$, $RO_2^{\cdot-}$, $HO^\bullet$, *etc*. However, some studies demonstrated cytotoxicity of AA mediated by production of H_2O_2 via synthesis of ascorbic radical from ascorbate [3,4,6]. This cytotoxicity was manifested when concentration of ascorbate radical attained a certain level [11]. It is known that production of H_2O_2 in cells strongly depends on NO level. NO in moderate concentrations can dramatically up-regulate production of O_2 and H_2O_2 via inhibition of mitochondrial respiration. High level of H_2O_2 can up-regulate activity of endothelial NO-synthase (eNOS) thereby elevating NO content [14]. Cell sensitivity to cytotoxic action of H_2O_2 is also determined by cell type [13]. Despite great interest to the problems of oxidative stress, the role of superoxide O_2^- and NO in pro- and antioxidant effects remains poorly understood.

Our aim was an intravital assessment of generation of oxygen radicals in the cell under normal conditions and after application of AA in various concentrations to reveal the factors disturbing free-radical balance and provoking cell death.

MATERIALS AND METHODS

The study was carried out on two cell lines expressing various members of NOS family: human endotheliocytes ECV 340 (eNOS) and human carcinoma cells HeLa G-63 (iNOS). We previously showed that these cell lines differed by intracellular NO level and modi-

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fiability of this level by of NO-synthases inhibitors [1]. Both cell lines were cultured in modified Eagle's medium (ICN) supplemented with 10% fetal serum (Biolot). The culture contained antibiotic gentamicin and modifiers of the cell redox status AA (Lumi) and catalase (MPBiomedicals Inc.). All reagents were prepared immediately before use.

Dihydroethidine (DHE, Sigma) was employed to determine the intracellular level of superoxide anion O_2^-. The cell suspension was dark-incubated with 5 μM DHE for 30 min at 37°C and then examined in a flow cytometer. 4,5-Diaminofluorescein diacetate (DAF-2DA, Cayman) was used as an NO indicator. The final concentration of the dye was 5 μM and the incubation time at 37°C was 30 min.

Cytofluorometry was carried out in a flow cytometer [1]. The flow cytometric assessment of cellular NO and superoxide levels was based on recording green fluorescence of triazolfluorescein and red fluorescence of ETD^+, respectively. At least 20 thousand cells were analyzed in each sample. The experiments were repeated no less than 5 times. The histograms below show one of such experiments. Fluorescence was recorded both in linear and semi-logarithmic (log_2) scales.

RESULTS

Comparative studies of the effects of AA in concentrations ranging from 0.5 to 20 mM on the intracellular levels of superoxide and NO was carried out on HeLa

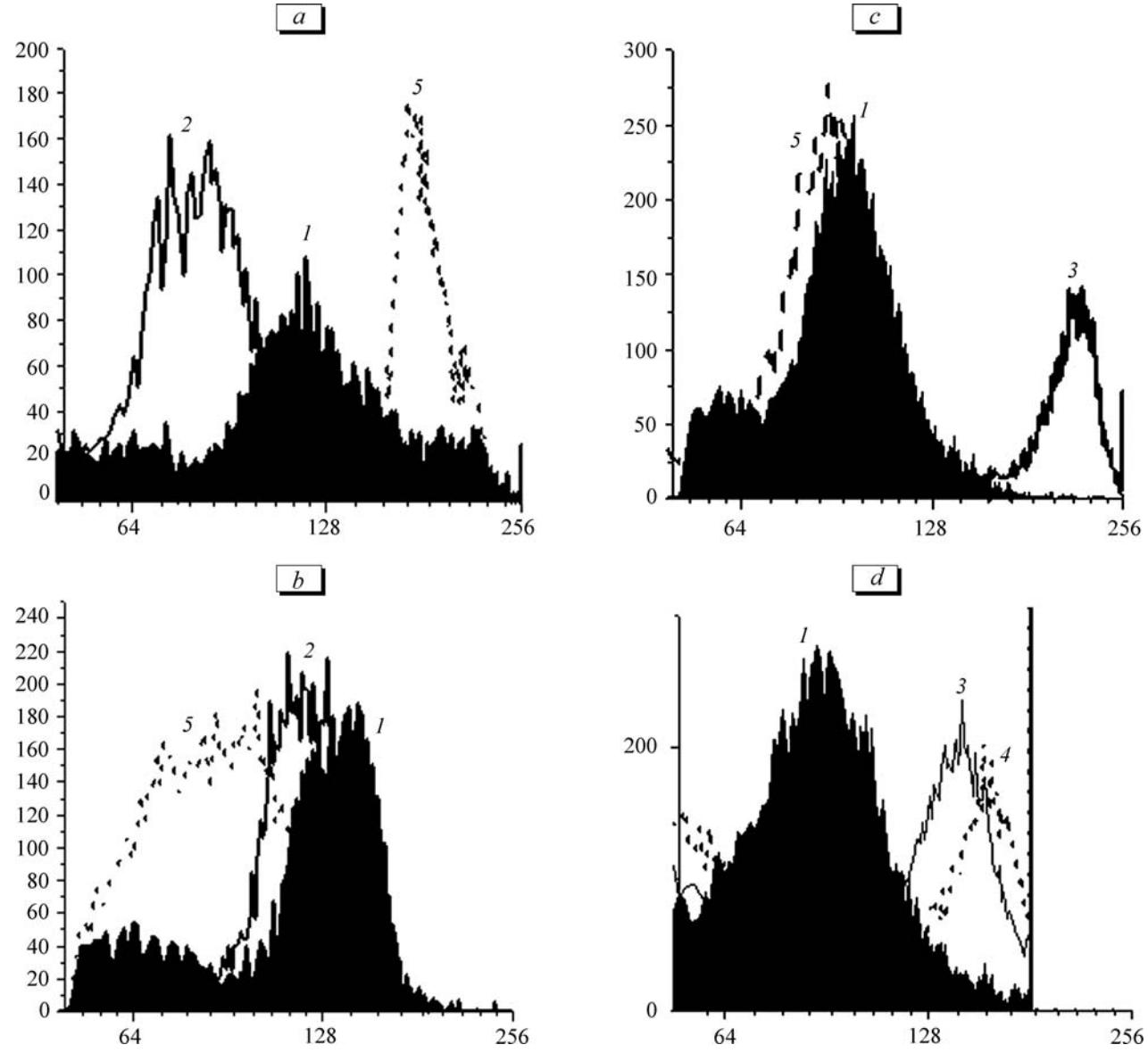

Fig. 1. Effect of AA in various concentrations on intracellular levels of superoxide O_2^- (*a*, *b*) and NO (*c*, *d*) in HeLa G-63 (*a*, *c*) and ECV 304 (*b*, *d*) cells. Abscissa: fluorescence channel number in semi-logarithmic scale (log_2). Ordinate: number of cells in relative units. The plots correspond to untreated cells (*1*) and cells treated with 1 mM (*2*), 5 mM (*3*), 10 mM (*4*), and 20 mM (*5*) AA.

G-63 and ECV 304 cell lines. Examination of AA effect on intracellular superoxide showed that one-hour exposure of the cells to AA in concentrations <10 mM decreased fluorescence intensity in comparison with untreated (control) cells. Further elevation of AA concentration decreased fluorescence in ECV 304 cells (Fig. 1, *b*). In contrast, fluorescence of HeLa G-63 cells increased with further elevation of AA concentration and surpassed the control value at 20 mM AA (Fig. 1, *a*). In the last case, elevation of concentration evidently changed the antioxidant properties of AA for the prooxidant ones. However, the anti- or prooxidant properties of AA depended on not only its concentration, but also cell type, since in contrast to HeLa G-63 cells, elevation of AA concentration to 20 mM in the experiments with ECV 304 cells continued to decrease the intracellular superoxide level.

Figures 1, *c* and 1, *d* show the effect of one-hour application of AA in various concentrations followed by incubation with DAF-2DA on intracellular NO level. As a result, the intensity of fluorescence increased in HeLa G-63 (*c*) and ECV 304 (*d*) cell lines, which attests to elevation of intracellular NO in comparison with untreated control cells. Elevation of AA concentrations in the range of 5, 10, and 20 mM decreased fluorescence intensity in HeLa G-63 cells, but increased it in ECV 304 cells. Thus, at moderate concentrations of AA (up to 10 mM), the decrease in in-

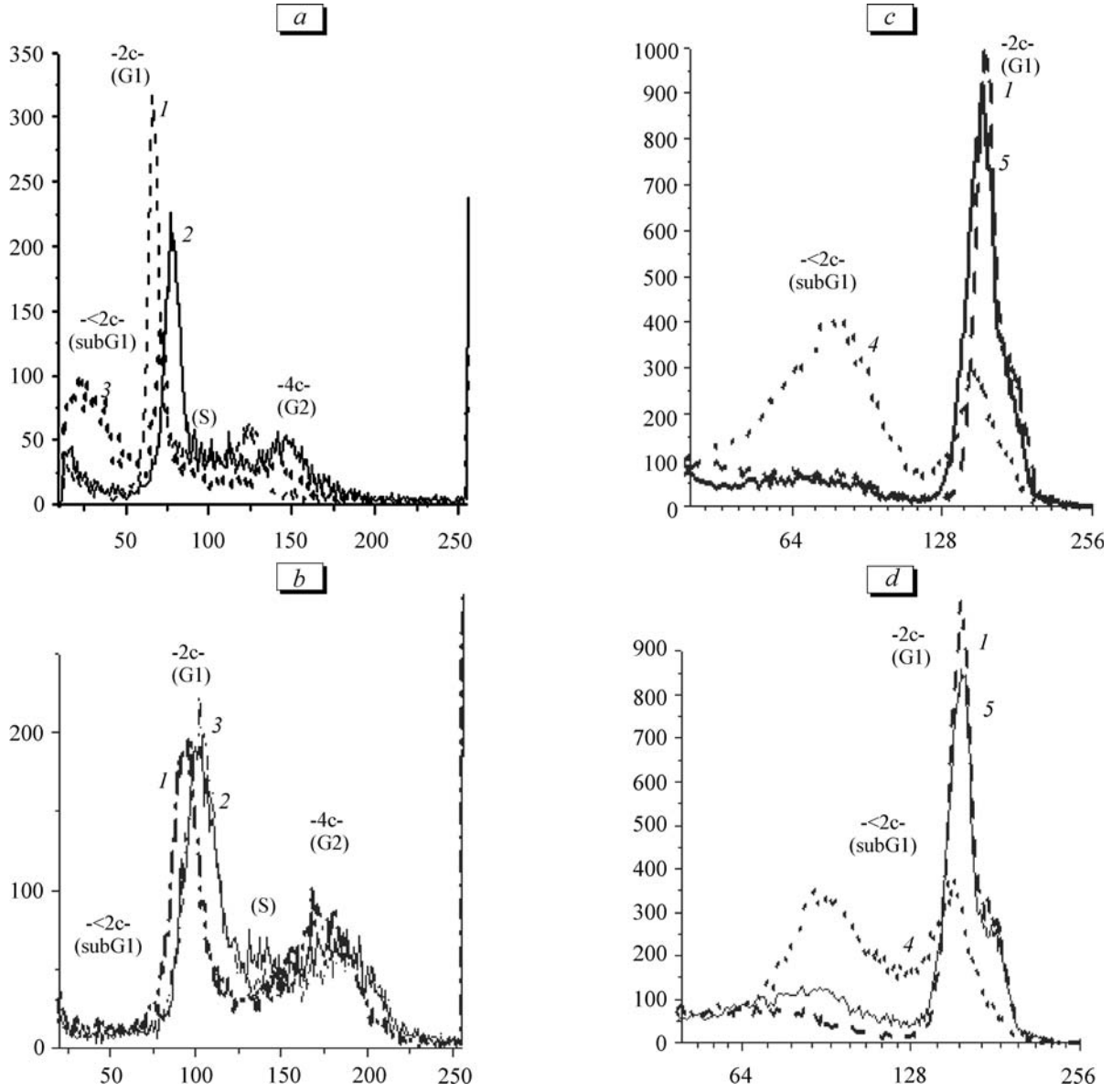

Fig. 2. Effect of AA on apoptotic death of HeLa G-63 (*a*, *c*, *d*) and ECV 304 (*b*) cells assessed by cell distribution over DNA content revealed by ethidium bromide staining. Abscissa: fluorescence channel number in linear (*a*, *b*) and $\log_2$ (*c*, *d*) scales. Ordinate: number of cells in relative units. *1*) untreated cells; *2*) 1 mM AA; *3*) 20 mM AA; *4*) 20 mM AA (*c*) or 30 mM AA (*d*); *5*) catalase (200 U/ml)+AA (20 or 30 mM).

tracellular superoxide concentration was paralleled by an increase in NO level. Moreover, application of 20 mM AA increased superoxide level and decreased NO content. The revealed negative correlation between intracellular levels of superoxide and nitric oxide attests not only to antioxidant effect of NO, but also to the balance between these radicals. These results agree with other data obtained in the experiments with different methods of free radical assay [2].

The AA concentrations used in this study (0.5, 1, 5, 10, and 20 mM) are considered as pharmacological. At such concentrations, AA produces greater cytotoxic action against the tumor cell lines in comparison with the normal cells [3-5]. In this study, we assessed the effects of AA in this concentration range on apoptotic death of HeLa G-63 and ECV 304 cells. The cells in the logarithmic growth phase were incubated for 1 h in a medium with AA concentrations ranging from 0.5 to 30 mM. Then the cells were washed from AA and incubated in complete growth medium for 21 h. Analysis of composition of the cell population was performed in the flow cytometer after the cells were harvested from the flasks with Versene solution and stained in hypotonic ethidium bromide solution. Exposure of the cells to 0.5 and 1 mM AA did not induce the apoptotic death in both cell lines. In contrast, the cell fate dramatically changed at greater AA concentrations starting from 20 mM. In this case, the histograms of HeLa cells (Fig. 2, *a*) clearly showed the sub-G1 peak, while similar cell subpopulation was not observed in ECV 304 cells (Fig. 2, *b*). The apoptotic death manifested itself not only by appearance of cell subpopulation with DNA content less than 2c, but also by morphological signs such as nucleus segregation into apoptotic bodies. Microscopy of HeLa G-63 and ECV 304 cells confirmed the cytometry data. Evidently, AA at high concentrations selectively triggered the apoptotic death of the tumor cells which agrees with previous reports [3,4,6,10,11]. The specific mechanisms of AA selective cytotoxicity are not comprehensively clear. This selective cytotoxicity can be explained by up-regulation of glycolysis in the tumor cells. Under conditions of enhanced glycolysis, vitamin C enters the cells more easily resulting in a larger sensitivity of cells to oxidative stress [15]. It is an established view that ascorbate cytotoxicity results from H_2O_2 generation [3]. However, recent study of HeLa cells demonstrated the effect of H_2O_2 in various concentrations (50 μM-1 mM) on cell cycle arrest during G_2 phase without signs of apoptotic cell death [9].

We assessed the effect of catalase on apoptotic death of HeLa cells induced by AA at high concentrations. The cells in the logarithmic growth phase were incubated in a medium containing catalase (200 U/ml) with AA in various concentrations. After 1 h, the growth medium was replaced with the standard one (without the above agents) for 21 h, thereafter flow cytometry was performed. To demonstrate the catalase effect more clearly, the semi-log (log_2) plots were used. This enzyme diminished the apoptotic death of cells induced by AA in concentrations of 20-30 mM to the control level (Fig. 2, *c*, *d*). Evidently, H_2O_2 plays an important role in AA-induced apoptotic death, which agrees with other studies [3,4,6].

Thus, we carried out the intravital examination of changes in intracellular levels of superoxide O_2^- and nitric oxide NO and demonstrated a negative correlation between these parameters depending on AA concentration. We also showed the potency of AA at high concentrations to induce apoptotic death in tumor cells. The differences in cytotoxicity of AA in high concentrations towards the human carcinoma cells and endotheliocytes were revealed.

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