

Short communication

**ASCORBIC ACID INHIBITS THE MIGRATION OF WALKER 256
CARCINOSARCOMA CELLS #**

EWA WYBIERALSKA, MONIKA KOZA, JOLANTA SROKA, JAROSŁAW
CZYŻ and ZBIGNIEW MADEJA*

Department of Cell Biology, Faculty of Biochemistry, Biophysics and
Biotechnology, Jagiellonian University, Gronostajowa 7, 30-378 Kraków,
Poland

Abstract: The results of several experimental studies have shown that ascorbic acid inhibits tumor growth and metastasis. Ascorbic acid is an antioxidant that acts as a scavenger for a wide range of reactive oxygen species (ROS). Both tumour metastasis and cell migration have been correlated with the intracellular ROS level, so it was postulated that the inhibitory effect of ascorbic acid derivatives on cell motility may be caused by scavenging of ROS. Time-lapse analyses of Walker 256 carcinosarcoma cell migration showed that both the speed of movement and the cell displacement were inhibited by ascorbic acid applied in concentrations ranging from 10 to 250 μ M. This effect correlated with a reduction in the intracellular ROS level in WC 256 cells, suggesting that ROS scavenging may be a mechanism responsible for the inhibition of WC 256 cell migration. However, another potent antioxidant, N-acetyl-L-cysteine, also efficiently decreased the intracellular ROS level in WC 256 cells, but did not

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* Author for correspondence; e-mail: zibi@mol.uj.edu.pl

Abbreviations used: Asc – ascorbic acid, DCF-DA – 2',7'-dichlorofluorescein diacetate; NAC – N-acetyl-L-cysteine; PBS – phosphate buffered saline; ROS – reactive oxygen species; WC 256 – Walker 256 carcinosarcoma

affect the migration of the investigated cells. These results demonstrate that intact, unmodified ascorbic acid applied in physiologically relevant and non-toxic concentrations exerts an inhibitory effect on the migration of WC 256 carcinosarcoma cells, and that this may be one of the factors responsible for the anti-metastatic activity of vitamin C. However, our data does not support the hypothesis that the scavenging of intracellular ROS is the main mechanism in the inhibition of cancer cell migration by ascorbic acid.

Key words: Ascorbic acid, Cell migration, Metastasis, Reactive oxygen species

INTRODUCTION

The effect of ascorbic acid on tumour development remains a controversial subject [1, 2]. However, the tumour growth-inhibiting effect of ascorbic acid has been demonstrated in several epidemiological and experimental studies [1, 3, 4]. While many reports have shown the cytotoxic effects of ascorbic acid on a number of malignant and non-malignant cell lines *in vitro* and *in vivo*, much less is known about its inhibitory effect on the formation of metastases by cancer cells [4-8]. Metastasis is a complex multistep process, and is a major cause of death amongst cancer patients. The induction of cancer cell migration is a key step in tumour metastasis, and a correlation between the motility of tumour cells and their metastatic potential has been demonstrated in several studies [9, 10]. Ascorbic acid (vitamin C) is a major water-soluble antioxidant that acts as a scavenger for a wide range of reactive oxygen species (ROS). As an electron donor, and therefore a reducing agent, it prevents other compounds from being oxidized [11]. Ascorbic acid is, among other effects, capable of regenerating vitamin E from the tocopheroxyl radical, which is formed as a result of the inhibition of lipid peroxidation by vitamin E [12]. It was also shown that ascorbic acid protects against DNA damage induced by hydrogen peroxide in human lymphocytes [13]. Moreover, it was demonstrated that the levels of hydroxyl radicals in cells treated with ascorbic acid derivatives were markedly diminished relative to those of non-treated cells as evaluated by the electron spin resonance method using the spin trapping agent DMPO [14]. The attenuation of intracellular reactive oxygen species by derivatives of vitamin C was also shown with the redox indicator CDCFH-DA [6]. However, although the antioxidant activities of vitamin C have been shown in several *in vitro* experiments, *in vivo* vitamin C treatment has not resulted in changes in the biomarkers of oxidation or in the clinical outcome [11]. In addition, ascorbic acid can exert a pro-oxidant activity under certain conditions, particularly in the presence of transition metal ions or alkalis [15]. Since both tumour metastasis and cell migration have been correlated with the intracellular levels of ROS, it was postulated that the inhibitory effect of ascorbic acid on cell motility may be caused by scavenging of ROS [6, 8, 16, 17]. However, it was demonstrated that the inhibitory effects were characteristic for ascorbic acid derivatives rather than for intact, unmodified ascorbic acid [6, 8].

The specific aim of this study was to establish if intact ascorbic acid inhibits the migration of invasive Walker 256 carcinosarcoma cells. We also attempted to verify the hypothesis that the inhibition of cancer cell migration by ascorbic acid is caused by scavenging of the intracellular reactive oxygen species.

MATERIAL AND METHODS

Cell culture

Walker 256 carcinosarcoma cells were cultured in RPMI-1640 medium (Sigma, St. Louis, MO/USA), supplemented with 5% heat-inactivated fetal calf serum (FCS; Gibco Lab., New York, USA), 100 I.U./ml penicilin, 10 µg/ml neomycin and 100 µg/ml streptomycin (Polfa, Tarchomin, Poland) in a humidified atmosphere with 5% CO₂ at 37°C [18].

Time lapse-monitoring of cell movement

Time-lapse monitoring of cell movement was carried out as described previously [19, 20]. WC 256 cells were plated to a Corning flask at a density of 20,000 cells/cm², and incubated in the culture medium in a humidified atmosphere with 5% CO₂ at 37°C for 24 hours. Subsequently, the culture medium was replaced and the cells were incubated for one hour in the presence of various concentrations of L-ascorbic acid (10-250 µM) or N-acetyl-L-cysteine (0.5-10 mM) (Sigma, St. Louis, MO/USA). Then, cell movement was recorded for 2 hours with a Hitachi CCD camera attached to an Olympus IMT-2 inverted microscope. In these experiments, we did not observe any significant decrease in the medium pH in the presence of 10-250 µM ascorbic acid. The tracks of individual Walker 256 carcinosarcoma cells were generated as described previously [19, 20]. Trajectories were constructed from 48 subsequent cell centroid positions recorded over 2 hours at time intervals of 2.5 minutes, and plotted in circular diagrams. Parameters characterizing cell locomotion were computed for each cell population as described previously [19, 20]. A total of 50 cells were analyzed for each value measured.

Cell viability

To determine the effect of ascorbic acid and N-acetyl-L-cysteine (NAC) on cell viability, WC 256 cells were plated to 6-well plates at a density of 10,000 cells/cm² and cultured for 24 hours. Then the cells were incubated for 3 hours with different concentrations of ascorbic acid or NAC. Thereafter, the cells were harvested with trypsin treatment, and the number of viable cells was determined by the fluoresceine diacetate and ethidium bromide test [21].

Measurement of reactive oxygen species

To measure the generation of ROS, WC 256 cells were incubated for 1 hour in the presence of different concentrations of ascorbic acid or NAC. Then the cells were loaded for 15 minutes with 2',7'-dichlorofluorescein diacetate (DCF-DA; Sigma, St. Louis, MO/USA) at a final concentration of 10 µg/ml in PBS

supplemented with 5.6 mM glucose and 1% FCS. At the end of incubation, the monolayer was rinsed with PBS and the cells were examined alive at 37°C under a Leica DM IRE2 fluorescence microscope equipped with a Leica DC350 FX digital camera and a fluorescein filter set. The integrated fluorescence intensity of at least 50 cells for each specific experimental treatment was measured with a Leica FW4000 image analysis system.

Statistical analysis

Each parameter was calculated as the mean and standard error of the mean (SEM). The statistical significance was determined by the t-Student test with $p < 0.05$ considered to indicate significant differences.

RESULTS AND DISCUSSION

Time-lapse analyses of cell migration were carried out in order to investigate the effect of ascorbic acid on the motility of WC 256 cells. Representative trajectories of cells moving under control conditions and in the presence of ascorbic acid are illustrated in circular diagrams in Fig. 1A. Analysis of the individual trajectories showed that both the speed of cell movement and cell displacement were inhibited by ascorbic acid in a dose-dependent manner (Fig. 1B). A statistically significant inhibition of migration was induced by 100 μM ascorbic acid. Interestingly, we did not observe any apparent effect of ascorbic acid on the morphology and polarization of WC 256 cells (data not shown).

This inhibitory effect was independent of cytotoxic activity, as ascorbic acid did not affect the viability of WC 256 in concentrations ranging from 10 to 250 μM (Fig. 2). We demonstrated that ascorbic acid may significantly inhibit the migration of invasive cancer cells at a physiologically relevant concentration which may be attained by oral administration. Ascorbic acid administered orally at a dose of 1.25 g produced mean peak plasma concentrations of $135 \pm 20.6 \mu\text{mol/l}$ and $885 \pm 201.2 \mu\text{mol/l}$ for intravenous administration [22]. Our results suggest that the anti-metastatic effect of ascorbic acid [5-8] may at least in part be due to inhibition of the migration of cancer cells. Similar results were obtained by Nagao *et al.* [6]; however, they observed significant inhibition of melanoma B16BL6 random movement by ascorbic acid-2-O-phosphate but not by intact ascorbic acid. The suppression of human lung carcinoma migration by ascorbic acid-2-O-phosphate-6-O-laureate was also observed by Liu *et al.* [8]. Here, we show that not only ascorbic acid derivatives but also intact, unmodified ascorbic acid inhibits the motility of cancer cells.

Increasing evidence indicates that at low levels, ROS can function as signaling molecules participating in the regulation of cell proliferation, migration and adhesion [17, 23, 24]. It was suggested that a decrease in the intracellular ROS level induced by ascorbic acid may be responsible for the inhibition of cell motility [6, 8]. To determine whether intracellular ROS levels correlate with the inhibition of WC 256 cell migration, we subsequently measured DCF-DA fluorescence in cells treated with various concentrations of ascorbic acid (Fig. 3).

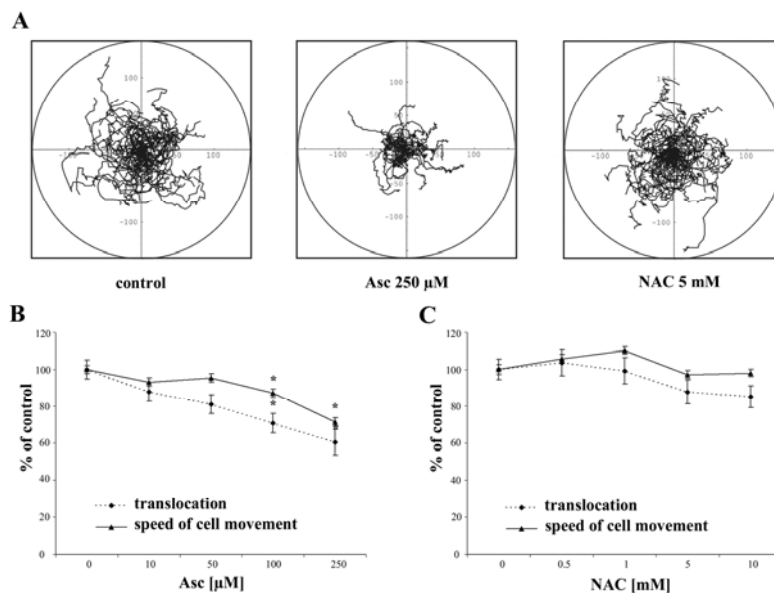


Fig. 1. The effect of Asc and NAC on the motility of WC 256 cells. A – Composite trajectories of migrating cells are displayed in circular diagrams drawn with the initial point of each trajectory placed at the origin of the plot. Each trajectory was constructed from 48 successive positions of the cell centroid recorded at 2.5-minute intervals. Scale bars: [μm]. B and C – Summary of the quantitative data showing the effect of Asc (B) and NAC (C) on the locomotion of WC 256 cells. For each value measured, 50 cells were analyzed. The values are the means from 3-4 independent experiments. The parameters translocation and speed of cell movement were presented as a percentage of the values for the control. Error bars represent SEM, * indicates statistically significant differences.

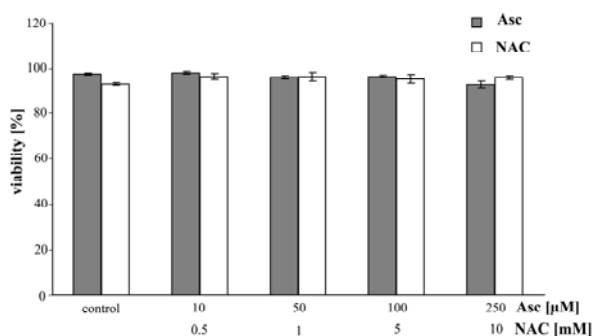


Fig. 2. The effect of Asc and NAC on the viability of WC 256 cells. The proportion of viable cells after 3 hours of incubation with Asc and NAC at various concentrations (10-250 μM and 0.5-10 mM, respectively) or with RPMI 1640 (as the control) was calculated on the basis of the fluorescein diacetate and ethidium bromide test. The data represents the average of three independent experiments, each performed in triplicate. Error bars represent SEM.

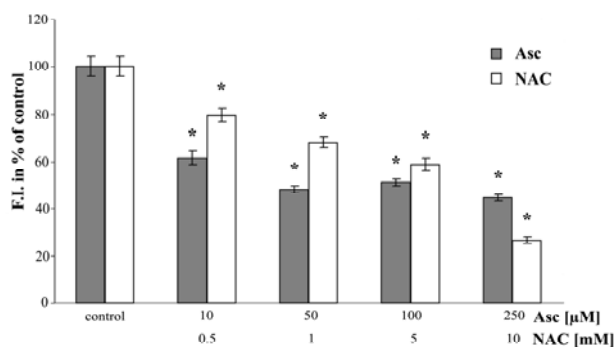


Fig. 3. Asc and NAC reduce the level of ROS in WC 256 cells. WC 256 cells were incubated in the presence of different concentrations of ascorbic acid or NAC. Then the cells were loaded with DCF-DA. Fluorescence intensity (F.I.) is presented as a percentage of the control value. The integrated fluorescence intensity of at least 50 cells was measured for each specific experimental treatment. The data represents the average of three independent experiments. Error bars represent SEM, * indicates statistically significant differences.

Our results demonstrate that ascorbic acid decreases ROS levels in WC 256 cells in a dose-dependent manner. This suggests that the scavenging of ROS may be responsible for the inhibition of cell migration. To test the hypothesis that a decrease in ROS levels in cancer cells leads to the inhibition of their motility, we investigated the effect of another potent antioxidant, N-acetyl-L-cysteine, on WC 256 cell migration (Fig. 1 A, C) and intracellular ROS levels (Fig. 3). An analysis of the cell trajectories showed that the motility of WC 256 cells was not affected by NAC in concentrations ranging from 0.5 to 10 mM. However, NAC applied at these concentrations efficiently decreased the intracellular ROS levels in WC 256 cells. Therefore, our results suggest that, at least in our model, a decrease in ROS levels is not sufficient to inhibit cancer cell motility.

The molecular mechanism of ascorbic acid activity with regard to the inhibition of cancer cell migration is not understood, and due to the broad biological activity of vitamin C, it is difficult to draw conclusions about the specific regulatory pathways involved in the inhibition of cell migration. It was suggested that a decrease in the intracellular ROS level induced by ascorbic acid derivatives may play an important role in the inhibition of migration, probably by interfering with redox-regulated signaling pathways [6, 8, 14]. However, our results indicate that the antioxidant effect of ascorbic acid is not by itself sufficient to inhibit cell migration. This discrepancy might have resulted from the different cell lines used in the experiments. As it was demonstrated that different cell types show different sensitivities to ascorbic acid [25], it is possible that WC 256 cells are more sensitive to this compound than the cell lines used in the previous experiments [6, 8, 14]. Moreover, we also cannot exclude that in our model, ascorbic acid derivatives would induce inhibition of WC 256 cell migration at a lower concentration than intact vitamin C. However, our data does

demonstrate that unmodified ascorbic acid may also be an efficient inhibitor of cancer cell migration. The observations of Liu *et al.* [14] that ascorbic acid derivative affects the localization and expression of the small GTP-binding protein RhoA suggests that the regulation of this protein by ascorbic acid may be involved in the inhibition of cell migration. However, the detailed mechanism of this regulation is not known. It was also demonstrated that enrichment of intracellular ascorbate content of tumour cells induces, via a post-transcriptional inhibition of MMP proenzyme production, a marked decrease in metalloproteinases 2 and 9, which are involved in the regulation of cell migration [6]. However, further experiments elucidating the mechanism of inhibition of cellular movement by ascorbic acid are required to get some insight into the underlying signalling and regulatory pathways.

In summary, our results demonstrate that intact, unmodified ascorbic acid in physiologically relevant and non-toxic concentrations exerts an inhibitory effect on the migration of WC 256 carcinosarcoma cells, and that this may be one of the factors responsible for the anti-metastatic activity of vitamin C. It was suggested that a decrease in the intracellular ROS level induced by ascorbic acid derivatives may play an important role in the inhibition of the migration and invasion of cancer cells [6, 8, 14]. However, our data does not support the hypothesis that the scavenging of intracellular reactive oxygen species is the main mechanism inhibiting cancer cell migration by ascorbic acid, because NAC, which also decreased the ROS level in WC 256 cells, did not affect their motile activity.

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