Tumor invasion is inhibited by phosphorylated ascorbate via enrichment of intracellular vitamin C and decreasing of oxidative stress

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Abstract Tumor metastasis and invasion were shown to be inhibited by the 2-O-phosphorylated form (Asc2P) of L-ascorbic acid (Asc); intact Asc did not inhibit tumor invasion when added once, but appreciably inhibited it upon repeated addition. The anti-metastatic effect is attributable to a marked enrichment of intracellular Asc by Asc2P, subsequently dephosphorylated. Asc2P scavenged most of the intracellular reactive oxygen species (ROS), and notably inhibited production of matrix metalloproteases and cell motility. ROS was decreased by Asc2P more markedly than by Asc added once. Thus, involvement of ROS in tumor invasion and a potent anti-metastatic therapy by ROS-decreasing agents are suggested.

Key words Ascorbic acid · Invasion · Metastasis · Reactive oxygen species · Matrix metalloprotease

Abbreviations Asc L-ascorbic acid · DehAsc dehydro-ascorbic acid · Asc2P ascorbic acid-2-O-phosphate · Asc intracellular ascorbic acid · MMP matrix metalloprotease · ROS reactive oxygen species · ROS intracellular ROS

Introduction

Considerable controversy has existed regarding megadose vitamin C therapy as a defense against cancer since the 1970s when Cameron and Pauling proposed it (Cameron and Pauling 1974). Two subsequent double-blind trials were carefully performed at the Mayo clinic (Moertel et al. 1985), showing no anticancer effect of vitamin C. Ambiguity in the effects of vitamin C is attributable either to its instability under physiologic conditions, such as neutral pH and 37 °C (Hata and Senoo 1989), or to its amphoteric action as the antioxidant and prooxidant (Paolini et al. 1999). The inherent defects of L-ascorbic acid (Asc) are considered, although not demonstrated, to be overcome by masking with phosphorylation of the C2-hydroxyl group in the 2,3-enediol moiety of an Asc molecule responsible for reduct ability; ascorbic acid-2-O-phosphate (Asc2P) as the resultant product (Hata and Senoo 1989) is thereafter esterified with cellular phosphates to persistently supply an intact form of Asc. Asc2P is superior to Asc in terms of promotion of collagen biosynthesis (Kurata et al. 1993) and scavenging of reactive oxygen species (ROS) (Fujiwara et al. 1997), whereas anticancer effects of Asc2P have not been shown.

Furthermore, tumor metastasis may be correlated to ROS (Mukai et al. 1987; Nonaka et al. 1993; Kundu et al. 1995; Tanaka et al. 1997) although this has not been demonstrated; still less explicable are metastatic inhibitors that exert a suppressive action against ROS. The present study has attempted to define the effects of vitamin C in cancer treatment using Asc2P, focusing on tumor invasion and metastasis stages.

Materials and methods

Cells

Human fibrosarcoma HT-1080 cells were obtained from the Japanese Cancer Research Resources Bank (JCRB). The highly metastatic substrain B16BL6 of mouse melanoma B16 cells were kindly provided by Dr. Isaiah J. Fidler, University of Texas.
Invasion and haptotactic cell migration assay

Invasiveness through reconstituted basement membrane Matrigel (Collaborative Research, Bedford, Mass.), or haptotactic migration toward fibronectin or laminin (Albrecht-Buehler 1977) on the lower surface of filters in a chemotactic chamber system was determined as previously described (Albin et al. 1987). HT-1080 or B16BL6 cells were incubated with or without Asc (Sigma) or Asc-2-O-phosphate magnesium salt (Asc2P, Showa Denko, Tokyo) for 18 h, and trypsinized to form a single-cell suspension in MEM + 10% dialyzed FBS) containing Asc2P, Asc or dehydroascorbic acid (DeAsc) at indicated concentrations, which was also added to the upper and lower compartments of a chamber. After 3.5 h of incubation, invasive cells adhering to the lower surface were fixed, stained and counted.

Pulmonary metastasis assay

B16BL6 cells were pretreated with or without Asc2P at 300 μM as similarly done for tumor invasion assay (Saiki et al. 1989). A portion (5×10^5/0.2 ml PBS) of cell suspension was inoculated via the tail vein into 9-week old female C57BL/6 mice (Simizu Laboratories Co., Japan). The mice were then intravenously administered, daily for five consecutive days or as a single dosage, with Asc2P, Asc-2-O-alpha-glucoside (Hayashibara, Okayama, Japan) dissolved in 0.2 ml PBS(−) or PBS(−) alone. For one experimental group, single-cell suspension was admixed with Asc2P, and immediately inoculated. After two weeks, the mice were killed, and their lungs were excised, rinsed, and fixed in Bouin’s solution, and surface metastatic foci were counted.

Intracellular Asc concentration

HT-1080 cells were administered with Asc2P or Asc as similarly done for invasion assay, crushed with a Potter-type Teflon homogenizer, and analyzed by HPLC and the coulometric ECD technique as previously described (Furumoto et al. 1998; Washko et al. 1992). Asc, Asc2P and DeAsc were separated as shown by retention times of 3.4, 6.5 and 20.0 min, respectively, under the chromatographic conditions employed.

Non-directional cell motility assay

Cellular random locomotion was quantified as previously described (Albrecht-Buehler 1977) with slight modification. B16BL6 cells (2–3×10^4) in a 6-well plate containing cover glasses coated with colloidal gold were incubated for 24-48 h in the absence or presence of Asc2P at indicated concentrations. Then, the cover glass was fixed with paraformaldehyde and then embedded in a gel/mount. Gold-free tracks where cells had been moving were observed under dark-field optics.

Gelatin zymography

The supernatant of the conditioned medium of HT-1080 cells cultured in the absence or presence of Asc2P or Asc at indicated concentrations for 18 h, was electrophoresed under non-reduced conditions in sodium dodecyl sulfate (SDS)-polyacrylamide gel containing 0.2% gelatin (8% total acrylamide) as previously described (Bullin et al. 1988). The gels were washed with 2.5% Triton X-100 for 1 h, incubated in 0.05 M Tris-HCl buffer (pH 7.5) containing 0.15 M NaCl, 0.01 M CaCl2 and 0.02% NaN3 for 12 h at 37 °C, and stained with Coomassie Brilliant Blue R250. In order to examine influences on the activation of MMP proenzymes, we also prepared supernatant of conditioned medium of HT-1080 cells and activated it by addition with Zn2+ in the presence or absence of Asc2P or Asc for 12 h at 37 °C.

In situ detection of cellular oxidative stress

Treatment of HT-1080 cells with Asc2P or Asc was carried out in the same way as for invasion assay. Cells were grown in the presence or absence of Asc2P, rinsed twice with PBS(−) and replaced by phenol red-free MEM containing 10% FBS and 10 μM dichlorodihydrofluorescein (DCDFH) (Molecular Probes, Eugene, Ore.) (Haugland 1996). After 60-min incubation, the supernatant was transferred into a new microplate to quantify the extracellular oxidative stress. Fresh medium without phenol red was added to the plate well for measurement of the intracellular oxidative stress. The fluorescence intensity was measured with a fluorescence plate reader CytoFluor 2350 (Millipore, Bedford, Mass.).

Electron spin resonance (ESR) spectroscopy

B16BL6 cells were incubated with Asc2P as similarly done for invasion assay, washed carefully with PBS(−) three times, and harvested with a rubber policeman. Cell suspensions were then lysed with Triton X-100 in PBS(−), and homogenized with a Potter-type Teflon homogenizer by ten strokes on the ice. Cell extract was mixed with a solution of the spin trapping reagent, 5,5-dimethyl-1-pyrroline-N-oxide (DMPO; Labotec, Tokyo), vortexed for 10 s, and immediately transferred to a quartz flat cell. ESR spectrum recording was started exactly 60 s after DMPO addition using an ESR spectrometer (X-band, JEOL JES-FR30) at room temperature (4 mW microwave power, 335.5 ± 5 mT modulation amplitude, and 1 s time constant). Reference samples of MgO: Mn2+ were used for determination of g-values and relative signal intensities. Ascorbyl radical (Asc') signal was observed as an asymmetric doublet with a line width of 0.18 mT centered at g = 2.007, in accordance with previously reported values (Morishige et al. 1983).

Statistical analysis

An experimental value is expressed as the mean ± SD. Student’s t-test was used to evaluate the significance of differences between groups, and the criterion of statistical significance was taken as P < 0.05.

Results

The inhibitory effect of Asc2P on tumor invasion

The invasive ability of human fibrosarcoma HT-1080 cells was assessed using the reconstituted basement membrane Matrigel to examine effects of Asc2P on the tumor invasion stage intrinsically commanding metastasis (Albin et al. 1987). Addition of Asc or DeAsc even at 300 μM, being higher than the normal human plasma concentrations of Asc, did not inhibit invasiveness. In contrast, addition of Asc2P at 250–300 μM inhibited invasion significantly (P < 0.05) as compared with the unadded control (Fig. 1a). Similar results were obtained for mouse melanoma B16BL6 cells (Fig. 1b). Effective concentrations of Asc2P used were as low as 5–7% of the lower threshold concentration (> 5000 μM) inhibiting tumor cell viability and growth (data not shown), indicating that invasion-inhibitory effects of Asc2P are not attributable to the direct cytotoxic action. Intact Asc was ineffective in inhibiting tumor invasion upon addition in a single-dosage manner, but effective upon intermittent additions of 2–4 times at intervals of 2–24 h, although not so markedly as Asc2P (Fig. 2), suggesting another important role of a constant intake of a natural form of vitamin C in everyday life. Because Asc is less effective than Asc2P in invasion assay