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Oxidative stress-mediated antitumor activity of erythorbic acid in high doses

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ABSTRACT

Intravenous (iv) infusion of high-dose ascorbic acid (AA) has been used as a treatment for cancer patients. The tumoricidal action of AA occurs due to its prooxidant effect. Erythorbic acid (EA), one of the AA epimers, has reduced vitamin C activity, while the antioxidant activity of EA is similar to that of AA. Currently, other physiological and pharmacological functions of EA are not well known. We examined the cytotoxicity of EA to murine colon carcinoma (colon-26) cells and the antitumor activity of EA in tumor-bearing mice. Cytotoxic activity of EA to colon-26 cells was evaluated by using the calcein-AM assay. EA showed the same cytotoxic activity to colon-26 cells as that of AA. The cytotoxicity of EA was shown to be caused by oxidative stress. Next, colon-26 tumor-bearing mice were iv administered EA and AA on alternate days for 4 times, and tumor growth rates were measured. Tumor growth was significantly inhibited by administration of high-dose EA in vivo as well as AA. Finally, the in vivo biodistribution and clearance of EA and AA were investigated in tumor-bearing mice. Endogenous AA in the tumor was consumed to resist oxidative stress caused by reactive oxygen species that was generated by administered EA. These results indicated that the oxidative stress-mediated antitumor activity is one of the pharmacological functions of high-dose iv EA.

1. Introduction

Ascorbic acid (AA, Fig. 1A) is a water-soluble compound known as vitamin C, and it is common knowledge that AA is an essential micronutrient for humans. AA shows various physiological and pharmacological activities, including collagen synthesis [1], drug metabolism [2], enhancing iron absorption [3] and antioxidant activity [4]. A number of recent publications report the prooxidant effects of antioxidants (e.g., resveratrol, quercetin, and curcumin) at high concentrations [5–7]. In the presence of transition metal ions such as iron and copper, high-concentration AA can exert prooxidant effects on a tumor, and it has been used by some practitioners as an intravenous (iv) infusion to treat cancer [8,9]. Intravenous infusion of high-dose AA temporarily increases the plasma concentration of AA in patients to a level 100–1000-times than that of healthy levels (from 50–100 μM to 25–30 mM) [10,11], while the upper limit value of oral administration was predicted to be approximately 220 μM [12]. Pharmacologic AA acts as a prooxidant agent. AA reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}), and Fe^{2+} acts as an electron donor to O_2, ultimately generating H_2O_2 in plasma and extracellular fluid. H_2O_2 then interacts with another transition metal to generate ROS, including the highly reactive hydroxyl radical [11,13–15]. In normal cells that have antioxidant enzymes such as catalase and glutathione peroxidase, ROS are immediately detoxified [14]. On the other hand, many cancer cells have low levels of several antioxidant enzymes [16]. The mitochondria in many cancer cells may have increased sensitivity to ROS. Therefore, mitochondria of cancer cells that have low anti-oxidant capacity are damaged by ROS [14], resulting in cell death due to a decrease in ATP production [14,17,18]. High-dose iv AA therapy exhibits a cytotoxic activity selectively to cancer cells and has fewer adverse effects than other cancer treatments.

Erythorbic acid (EA, Fig. 1A), a stereoisomer of AA, only differs from AA in the relative position of the hydrogen and hydroxyl groups on the fifth carbon atom. EA shows chemical properties similar to those of AA; however, the antiscorbutic activity of EA has been reported to be about one-twentieth of that of AA in vivo [19] and EA has only one-eighth of the activity of AA for stimulating collagen synthesis in vitro [20] because of the difference in quantity of intracellular uptake. It is believed that EA has a negligible vitamin C activity. On the other hand, EA has the same level of activity as that of AA for stimulating proline hydroxylation reaction [20]. Also, EA administration enhances iron absorption from Fe^{2+} sulfate more than does AA [21]. The antioxidant activity of EA is similar to that of AA, and EA has been widely used as an

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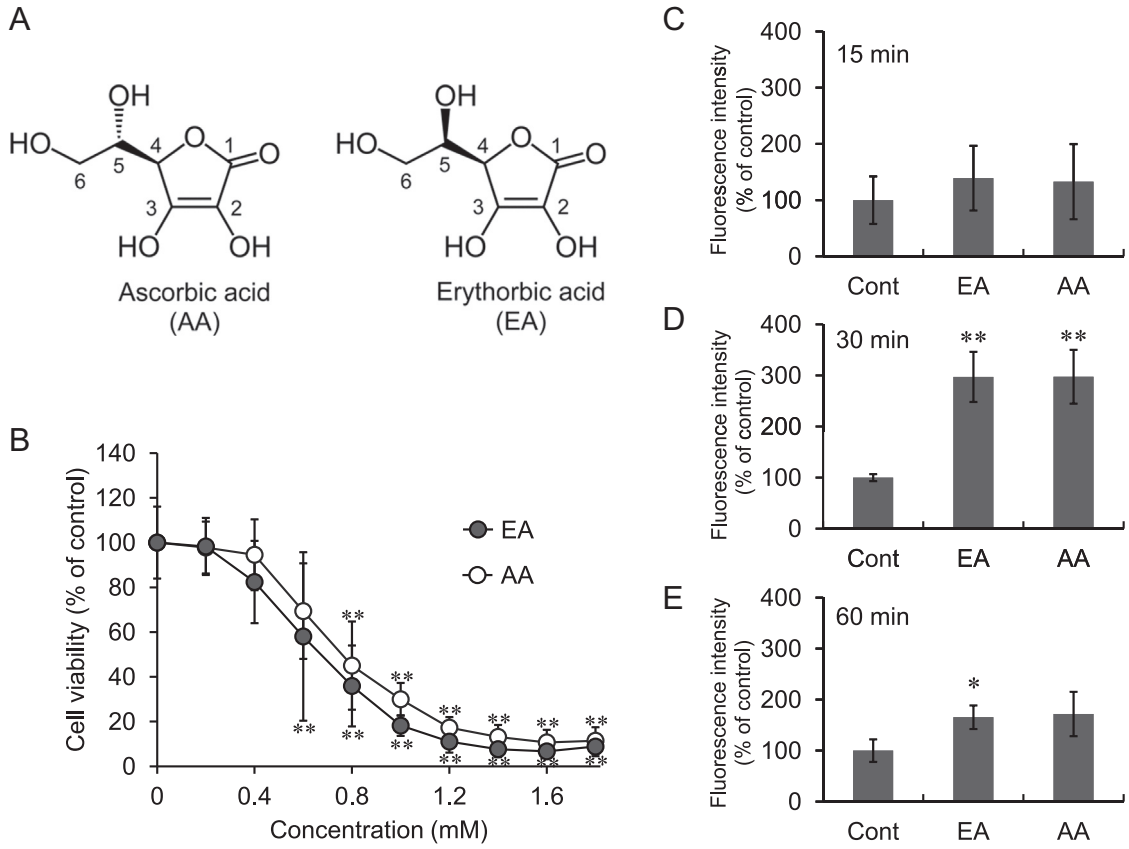

Fig. 1. Structures and oxidative stress-induced cytotoxicity of AA and EA. (A) Structures of AA and EA. (B) In vitro cytotoxicity of EA and AA. Colon-26 cells were incubated in a medium containing EA or AA at indicated concentrations for 24 h. Vehicle-treated cells were arbitrarily set as 100% control viability. (C)–(E) Amount of ROS generated by EA or AA in colon-26 cells. They were then treated with EA or AA (2 mM) for indicated times (C, 15 min; D, 30 min; E, 60 min). Results are expressed as percentage (%) relative to each control value. All data represent means ± SD of three independent cultures ($^{*}P < 0.05$; $^{**}P < 0.01$, compared to control).

antioxidant in many processed foods [19,21]. However, little is known about EA beyond these physiological functions.

Since EA has antioxidant activity similar to that of AA, it can be expected that high-dose iv EA will also act as a prooxidant like AA. However, there have been few studies in which the anticancer activity of high-dose iv EA was investigated. Administration of a relatively low dose of AA or EA intraperitoneally to a human mammary tumor xenograft mouse was reported not to inhibit tumor growth. However, when cupric sulfate was added to the injection fluid, tumor growth was depressed [22]. The aim of the present study was to find a new pharmacological function of EA. For this purpose, we assessed the antitumor activity of high-dose iv EA to murine colon carcinoma cells (colon-26) and cancer model mice. We also investigated the in vivo biodistribution and clearance of EA in tumor-bearing mice.

2. Materials and methods

2.1. Chemicals

Sodium erythorbate monohydrate (EA-Na · H_2O) was obtained from Tokyo Chemical Industries (Tokyo, Japan). Sodium ascorbate (AA-Na), heparin, calcein-AM solution and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). RPMI 1640 medium was purchased from Corning (NY, USA). Fetal bovine serum (FBS, heat-inactivated) was from HyClone (Logan, UT, USA). Triton X-100 was from Sigma (St. Louis, MO, USA). Dithiothreitol (DTT) was obtained from Nacalai tesque (Kyoto, Japan).

2.2. Cell lines

Murine colon carcinoma (colon-26) cells were purchased from RIKEN BRC CELL BANK (Tsukuba, Japan). Colon-26 cells were grown in RPMI 1640 with 10% FBS, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in 5% CO_2. Experiments were performed when cell growth was approximately 80% confluent.

2.3. Evaluation of in vitro cytotoxicity of EA

Cytotoxicity of EA to colon-26 cells was assessed using calcein-AM. The cells were suspended in RPMI 1640 medium and seeded in a 96-well microplate at a density of 1.0×10^4 cells/100 μL/well and then incubated for 24 h at 37 °C in 5% CO_2. After incubation, each medium was replaced with 90 μL of fresh medium and then 10 μL each of AA-Na and EA-Na were added and the cells incubated for 24 h. The cells were then washed with 100 μL of PBS(-) and added 100 μL of calcein-AM solution (5 μM). After 30 min of incubation, 20 μL of 0.6% Triton X-100 solution was added to each well. The fluorescence intensity (FI) of the cell lysate was recorded on a microplate reader (Varioskan Flash from Thermo Scientific, Ex. 485 nm, Em. 527 nm). Cell viability (%) was caluculated as (FI of treated-FI of blank)/(FI of control-FI of blank) × 100. The difference in cell viability between the control and treatment was analyzed by Dunnett's test ($^{**}P < 0.01$).

2.4. DCFH-DA assay

The relative amount of reactive oxygen species (ROS) to the control value was assessed by using DCFH-DA. Colon-26 cells were