Protective Effects by Dehydroascorbic Acid through an Anti-Oxidative Pathway and Toxic Effects by Ascorbic Acid through a Hydrogen Peroxide-Dependent Pathway in Tumor Cell Lines

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ABSTRACT | High concentrations of ascorbic acid (AA) exert pro-oxidative actions and inhibit tumor metastasis. The toxicity of AA centers on the generation of H_2O_2. But it remains largely unknown which process is inhibited by AA during the metastasis. The present study was designed to identify which process is suppressed by AA during the metastasis by use of malignant tumor cells lines. For this objective, we compared reduced and oxidized forms of AA in terms of cellular survival and levels of reactive oxygen species (ROS) in vitro. AA has ability to kill malignant tumor cells not binding to extracellular matrix. Once the cells detach from primary tumors, high concentrations of AA may drive them into a cell death pathway. The present results suggest that AA itself is toxic and that an oxidized form is protective. Thus, the reported neuroprotective effects may be mediated by an oxidized form, dehydroascorbate (DHA). This may be a therapeutic agent because it can penetrate the blood-brain-barrier. DHA protected the cells against oxidative stress by an anti-oxidative pathway. We did not find any protective effects by AA itself although it decreased levels of ROS much more than the oxidized form. Rather, AA induced potent toxic effects. It had selective toxic effects on non-attached cells. These selective toxic effects were suppressed by the presence of catalase, suggesting that H_2O_2 generation is involved in the cell death.

KEYWORDS | Ascorbic acid; COS7 cells; Dehydroascorbic acid; Glucose transporter 1; Hela cells; Hydrogen peroxide; HT22 cells; Isoascorbic acid; Reactive oxygen species; T98G cells

ABBREVIATIONS | AA, ascorbic acid; AFR, ascorbic acid free radical; CNS, central nervous system; CSF, cerebrospinal fluid; DCFH-DA, 2’,7’-dichlorodihydrofluorescin diacetate; DHA, dehydroascorbic acid; DHAR, GSH-dependent DHA reductase; GLUT1, glucose transporter 1; GSH, reduced from of glutathione; IAA, isoascorbic acid; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; ROS, reactive oxygen species; SD, standard deviation; SVCT2, sodium-dependent vitamin C transporter 2
1. INTRODUCTION

Accumulation of reactive oxygen species (ROS) is one of the major mechanisms of aging, neurodegeneration, and inflammation [1, 2]. Thus, antioxidants are considered to delay the pathological process of chronic diseases [1, 2]. Ascorbic acid (AA in Figure 1A) has been classified as a broad-spectrum antioxidant because it reacts with a wide array of ROS during various physiological and pathophysiological processes [3, 4]. In addition, AA can regenerate vitamin E, thereby protecting cells from lipid peroxidation. Thus, AA can maintain a balanced intracellular redox state and consequently protect cells from oxidative stress-induced damage [1, 2]. AA is involved in the first line of antioxidant defense, protecting lipid membranes and proteins from oxidative damages [3, 4]. AA functions both as an anti-oxidant and as a pro-oxidant [5–8]. These differential effects may depend on its concentrations [5–8]. According to the previous reports, the threshold seems to be around 0.1 mM [9, 10]. Below 0.1 mM, AA is protective against oxidative stress through an anti-oxidative pathway. Above 0.1 mM, AA is toxic by generation of hydrogen peroxide (H$_2$O$_2$) [5–8]. AA keeps Fe$^{2+}$ state by reduction of Fe$^{3+}$, which enhances the reaction. Thus, AA can kill malignant tumor cells and the infusion of high concentrations of AA may be an alternative medicine against tumors [5–8].

Many papers have reported that DHA and AA are absorbed along the entire length of the human small intestine, with a sodium-independent mechanism distinct from AA transporter [11, 12]. AA enters cells via sodium-dependent vitamin C transporter 2 (SVCT2), which is expressed in most human tissues [11, 12]. DHA competes with glucose for uptake via glucose transporter 1 (GLUT1) [11, 12]. Once inside the cell, DHA is again reduced to AA by a specific enzyme via the use of reduced from of glutathione (GSH), namely, the GSH-dependent DHA reductase (DHAR), and thereby trapped intracellularly [13, 14]. The rapid intracellular reduction of transported DHA to AA would maintain a favorable gradient for DHA entry [13, 14].

The most AA-condensed tissue is the brain, which contains about 25% of total AA. The major route by which AA enters the central nervous system (CNS) involves transport from the plasma to the cerebrospinal fluid (CSF) across the epithelium of the choroid plexus [9, 10]. This trans-cellular transport generates a 2–3-fold plasma-to-CSF AA gradient, resulting in CSF concentrations of about 0.2–0.4 mM, compared to plasma concentrations of 0.6–0.8 mM [9, 10]. The interactions between astrocytes and neurons are of special interest in terms of AA and DHA regulation in the brain [3, 4]. Astrocytes release AA through an anion channel as well as GSH precursors for neuronal redox regulation [15, 16]. Data indicate that AA is distinctly compartmentalized between neurons and astrocytes, with an average intracellular concentration of 10 mM in neurons and 1 mM in glial cells [3, 4]. Astrocytes have high redox capacity such as
ROS

GSH and are highly resistant to oxidative stress [15, 16]. The astrocytic redox power (GSH) is also important to AA-DHA metabolism [3, 4]. Because astrocytes absorb DHA through a GLUT1 pathway, DHA can be reduced to AA at the expense of GSH and be released to CSF. This AA accumulates into neurons through a SVCT2 pathway [11, 12]. Thus, we used neuronal HT22 and astrocytic T98G cells, as well as malignant tumor cells (COS7 and HeLa cells) to investigate the effects of AA and its derivatives.

Papers suggest that DHA and AA are protective in vivo against oxidative stress in the brain [3, 4]. DHA,

FIGURE 1. Chemical structures of AA, AFR, DHA, and IAA (A) and proposed mechanism of toxic effects by AA (B). AA and IAA have the common chemical structure of the “endiol” (reduced form) indicated by the dotted circle, which can give electron(s) to other molecules (A). Proposed mechanism of preferential formation of AFR and H₂O₂ in extracellular space [20] (B). In extracellular space, high concentrations of AA lose one electron and form AFR. The electron reduces a protein-centered metal: An example reaction is shown as reduction of Fe³⁺ to Fe²⁺. Fe²⁺ donates an electron to oxygen, forming active oxygen including superoxide with subsequent dismutation to H₂O₂. Catalase suppresses the toxic effects because of removing H₂O₂.
as well as AA, could crosses the blood-brain barrier [3, 4, 17] through transporters such as GLUT1 and SVCT2 and prevent cells against oxidative damage by increasing GSH levels [18, 19]. In fact, DHA has been reported to have protective effects as an antioxidant in experimental neurological disease models such as stroke, and DHA administration attenuates oxidative stress markers and inflammation in stroke models [18, 19]. DHA is proposed to be a therapeutic agent against neurodegenerative diseases such as ischemic damage as reported elsewhere [18, 19].

The most mysterious point of AA action is why antioxidant AA is toxic to cancer cells [5–8]. Many papers indicate that generation of H\textsubscript{2}O\textsubscript{2} is the center of issue [5–8]. High concentrations (over 0.1 mM) of AA induced selective death of cancer cells via the formation of ascorbic acid free radical (AFR) and H\textsubscript{2}O\textsubscript{2} in cell culture media. High concentrations of AA increase the levels of AFR in extracellular space (Figure 1B) [20]. In this case, the presence of high concentrations of AA in the extracellular fluid can induce death of cancer cells [20]. The electron lost from AA would reduce a protein-centered metal, selectively driving H\textsubscript{2}O\textsubscript{2} formation in the extracellular space [20].

In the present study, we used AA, isoascorbic acid (IAA), and DHA as shown in Figure 1A. IAA and AA share a reductive group (endiol), which is supposed to reduce Fe\textsuperscript{3+} to Fe\textsuperscript{2+} (Figure 1A). They are supposed to have the same chemical reaction (Figure 1B) [20] and to induce the same biological actions. We intended to compare these AA derivatives in terms of cellular protective effects against H\textsubscript{2}O\textsubscript{2} and toxic effect in various cell lines and the regulation of ROS. By these experiments, we confirmed that AA and IAA had almost the same toxic effects to cancer cells, suggesting that the toxic effects are due to “endiol” chemical structures. AA and IAA had no protective effects against H\textsubscript{2}O\textsubscript{2} although they reduced ROS. In other words, AA and IAA were potent antioxidants and toxic to cancer cells. In contrast, DHA had protective effects against H\textsubscript{2}O\textsubscript{2} and reduce ROS, suggesting that DHA acts as a typical antioxidant and is protective although it is not chemically an antioxidant. Possible mechanisms of the toxic effects by AA and protective effects by DHA are presented in the present work.

In addition, we focused on the toxic effects on non-attached cells because many papers showed that cancer cells have lower ability of cellular attachment to extracellular matrix [21–23]. AA has been reported to prevent cellular detachment from an original tissue and attachment onto distant organs during cancer metastasis [24, 25]. The supply of Fe\textsuperscript{2+} may be involved in the cellular attachment process AA enhances the supply of Fe\textsuperscript{2+} to hyper-activate the proline hydroxylase, which leads to the loss of protein conformation and results in the inhibition of cellular attachment process [26, 27]. Previous papers showed that AA may interfere and activate these process [28–30]. However, it is not known whether the toxic effects by AA are specific to attached cells or not. Thus, we compared the potency of toxic effects of AA between attached and non-attached cells. In the present study, we found that AA and IAA had more potent toxic effects on non-attached cells than on attached cells at least at ~0.1 and 0.3 mM. On the other hand, DHA did not have such selective toxic effects on non-attached cells, suggesting that these selective toxic effects to non-attached cells originate from “endiol” chemical structures of AA and IAA. The toxic effects of AA and IAA were suppressed by the presence of catalase, suggesting that the generation of H\textsubscript{2}O\textsubscript{2} plays a critical role in the toxic effects to non-attached cells as well as to attached cells.

2. MATERIALS AND METHODS

2.1. Chemicals

AA, DHA, and IAA (sodium salt), catalase, hydrogen peroxide (H\textsubscript{2}O\textsubscript{2}), and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Wako Junyaku (Tokyo, Japan). Stock solutions of AA, DHA, and IAA (sodium salt, 100 mM or 1000 mM) were prepared in Ca\textsuperscript{2+}, Mg\textsuperscript{2+}-free phosphate-buffered saline (Invitrogen, Carlsbad, CA, USA). 2,7\textsuperscript{′}-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma–Aldrich, St. Louis, MO, USA) stock solution (10 mM) was prepared in dimethyl sulfoxide and used at 10 µM in the culture medium.

2.2. Cultures of HT22, COS7, HeLa, and T98G Cells

HT22, COS7, HeLa, and T98G were cultured as described elsewhere [31–33]. HT22 and T98G cells were used as neuron- and glia-like cells, respectively [31–33]. Glial (T98G) cells have been reported to
have potent resistance to oxidative stress because they have high GSH metabolism [31–33]. COS7 and HeLa cells were used as simian and human malignant tumor cell lines, respectively. These cells were maintained in 10-cm dishes (Invitrogen, Carlsbad, CA, USA) containing 10 ml of Dulbecco’s Modified Eagle medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (Invitrogen, Carlsbad, CA, USA). The cells were seeded into 24-well plates at a density of 4 × 10⁴ cells/cm². When examining the toxic effects by compounds themselves on non-attached cells, the compounds were added just after the spreading of the cells into 24-well plates.

2.3. Effects of DHA, AA, and IAA

When examining the toxic effects by compounds themselves on attached cells, the compounds were added 24 h after the spreading of the cells into 24-well plates. When examining their effects on the H₂O₂ toxicity, the AA derivatives were added to the cultures after a 24-h incubation. In these cells, H₂O₂ caused cell death at 0.1–0.5 mM. One hour later, 0.1,
0.2, or 0.5 mM of H$_2$O$_2$ was added, and the cells were then incubated for an additional 24 h. To evaluate survival of the HT22 cells, we performed the MTT assay [31–33].

2.4. Dichlorofluorescein (DCF) Assay

The extent of cellular oxidative stress was assessed by monitoring the formation of free radical species by using DCFH-DA, as described elsewhere [32, 33]. Cells were plated 24 h before initiation of the experiment at a density of $4 \times 10^4$ cells/well in 24-well plates. AA derivatives and 0.01 mM DCFH-DA were added to the cells 30 min before the measurement. The plate was set into a Spark10M (Tecan Japan, Tokyo) under an atmosphere of 5% CO$_2$ and temperature of 37°C. 0.05 mM of H$_2$O$_2$ or vehicle was added to wells at 30 min, and the cells were incubated further for 180 min. DCF fluorescence was measured at a 485 nm (excitation wavelength) and 538 nm
ROS (emission wavelength) at 10-min intervals. Fluorescence values were expressed as a percentage of the value for the untreated control.

2.5. Statistical Analysis

Experiments presented herein were repeated at least 3 times with each experiment performed in quadruplicate. Data were presented as the mean ± standard deviation (SD). The statistical significance of differences was examined by performing Student’s t-test.

3. RESULTS

3.1. Protective Effects of DHA through an Antioxidant Pathway

At first, we compared reduced and oxidized forms of AA (AA, IAA and DHA) in terms of cell death and levels of ROS in vitro. We examined whether AA derivatives are protective against oxidative stress in HT22 and COS7 cells. H₂O₂ induced significant toxic effects at 0.1–0.5 mM (Figure 2A and 2B). The
presence of DHA, which has been used as a negative control of AA in many studies [1–4], significantly protected these cells against H₂O₂ (0.1–0.5 mM)-induced injury at 3 mM as shown in Figure 2A and 2B. Next, we examined whether these protective effects are associated with the levels of ROS (Figure 2C and 2D). H₂O₂ (0.05 mM) produced a significant increase in ROS both in HT22 and in COS7 cells. The presence of DHA (3 mM) reduced the levels of ROS activated by H₂O₂. Because the presence of DHA, AA, or IAA without H₂O₂ did not change the levels of ROS, the data were omitted from the Figures 2–4C and 2–4D. Thus, the protective effects by DHA were associated with a decrease in the levels of ROS induced by H₂O₂, suggesting that DHA acts as an antioxidant.

AA (Figure 3C and 3D) and IAA (Figure 4C and 4D) reduced the levels of ROS much more dramatically than DHA did. AA (1 mM) itself significantly enhanced cell death (Figure 3B) and AA (0.3 mM) did not affect the H₂O₂-induced cells death (data not shown). IAA did not protect the cells against H₂O₂ toxicity (Figure 4A and 4B), either, suggesting that AA and IAA should have other potent toxic actions.
3.2. Selective Toxicity of AA and IAA on Non-Attached Cells

The toxic effects of AA derivatives were investigated in HT22 (A), T98G (B), COS7 (C), and HeLa (D) cells in order to identify the mechanism of toxic effects of them. Various concentrations of DHA, AA and IAA were added to the cultures of attached (white columns) and non-attached (black columns) cells as shown in Figures 5–7, respectively. DHA induced toxic effects at high concentrations (e.g., 10 mM) (Figure 5). The potency of toxic effects on attached and non-attached cells was almost the same in HT22 (A), T98G (B), COS7 (C), and HeLa (D) cells (Figure 5).

AA induced toxic effects at similar concentrations on attached cells (white columns in Figure 6) at 3–10 mM as DHA did (white columns in the Figure 5). However, the toxic effects on non-attached cells (black columns in the Figure 6) were more potent...
than on attached cells (white columns in the Figure 6). IAA had similar results with AA (Figure 7). These results suggest that AA and IAA, but not DHA, are highly toxic to the process of cellular attachment. Because the chemical difference between DHA and AA/IAA exists in oxidative and reductive forms, these toxic effects may be linked with the reductive power. T98G cells were highly resistant to these compounds possibly because of high GSH metabolism [15, 16]. But the pattern of the results was almost the same as other cell lines (Figures 5B, 6B, and 7B).

### 3.3. AA and IAA Damaged Non-Attached Cells through a H₂O₂-Dependent Pathway

The toxic effects of AA derivatives themselves were examined in COS7 and HeLa cells (Figure 8). Especially, AA and IAA were found to have potent toxic effects on non-attached cells as shown in Figures 6 and 7.
Because AA has been reported to function both as anti-oxidant and as pro-oxidant pathways in tumor cells [5–8], the effect of 20 µg/ml catalase was examined in order to evaluate the roles of H₂O₂ release in the toxic effects of AA and IAA on non-attached COS7 and HeLa cells. In the cells treated with DHA (1–10 mM), the presence of catalase did significantly alter the toxic effects by a high concentration of DHA in COS7 cells, but not at all in HeLa cells. In addition, the presence of catalase did prevent the toxic effects of AA and IAA on non-attached COS7 cells, too, suggesting that upregulation of H₂O₂ was involved in the toxic effects of AA and IAA on non-attached cells.

### 4. DISCUSSION

#### 4.1. Toxic Effects of AA

Here we reported that reduced forms of AA derivatives (AA and IAA) selectively killed malignant tumor cells (COS7 and HeLa cells) not binding to extracellular matrix. Low concentrations (< 1.0 mM) of AA and IAA induced death of non-attached cells (Figures 6 and 7) although they killed the cells of attached cells at higher concentrations (> 1.0 mM), suggesting that AA and IAA have more potent toxic effects on the cells not binding to extracellular matrix. In Figure 8, the presence of catalase significant-
ROS

4.2. Protective Effects of DHA

The present results directly indicate that DHA is protective (Figure 2) and that AA is toxic (Figure 6), at least, at the limited concentrations (1.0–3.0 mM). DHA protected the cells against H$_2$O$_2$ toxicity associated with the levels of ROS (Figure 2), suggesting that the basic action of DHA is protective as an antioxidant. We proposed a possible protective mechanism by DHA (Figure 9B). The astrocytic T98G cells are resistant to pro-oxidative effects by AA and IAA and much more to toxic effects by DHA (Figure 5). This may be of physiological relevance to the high capacity of the reductive battery of astrocytes [15, 16]. The cells can uptake DHA and reduce it to AA and release AA for neurons [15, 16]. For this pathway, astrocytes have large capacity of GSH and high resistance to DHA [16]. Because AA is easily oxidized to DHA at a physiological pH, additional dietary intake of AA may not be able to increase the concentrations of DHA by stringent control of plasma AA levels and may increase DHA levels [9, 10].
The in vivo neuroprotective effects by DHA against ischemia may be mediated by DHA [18, 19]. DHA, as well as AA can penetrate the blood-brain barrier [17]. DHA is taken up by astrocytes and reduced to AA [15, 16]. AA is released to CSF and taken up by neurons [15].

5. CONCLUSION

We found that AA induced selective toxic effects on non-attached cells. These selective toxic effects were suppressed by the presence of catalase, suggesting that H$_2$O$_2$ generation plays a role. AA can kill malignant tumor cells not binding to extracellular matrix. Malignant tumor cells easily detach from extracellular matrix because of their weak binding to the matrix. AA has been reported to inhibit the metastasis of malignant tumors. The use of high-dose of AA in treating cancer patients demonstrated beneficial effects for inhibition of metastasis of malignant tumors.

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REFERENCES


