

Physiological Concentrations of Ascorbic Acid Potentiate Cell Death by Hydrogen Peroxide and Nitric Oxide of Non-Attached Cancer Cell Lines for the Possible Clearance of Cancer Cells from the Microcirculation

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ABSTRACT | Vitamin C (ascorbic acid, AA) exerts pro-oxidative actions and inhibits cancer metastasis, although AA is most famous for its antioxidant status. In this context, the physiological significance of the effect of AA at physiological concentrations ($< 100 \,\mu$ M) on cancer cells is largely unknown. Here, we found that such concentrations of AA significantly potentiated the death of non-attached cancer cells caused by hydrogen peroxide (H₂O₂) or a nitric oxide (NO) donor. In order to examine the involvement of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in this effect, we used an ROS- and RNS-sensitive fluorescent indicator, respectively. Even such low concentrations of AA negated the increase in ROS or RNS levels induced by H₂O₂ or the NO donor. In contrast, the oxidized form of AA, i.e., dehydroascorbic acid (DHA), did not affect the cell death. These results suggest that the reductive power of AA ("endiol") was closely linked with the AA-induced potentiation of cell death. Because the production of H_2O_2 and NO by endothelial cells is activated by the attachment of malignant cancer cells to these cells, these oxidants can clear cancer cells under coordination with the physiological concentrations of AA. This clearance may be the defense mechanism against cancer metastasis to distal organs used by AA and H₂O₂/NO at the first attachment of cancer cells to the vascular endothelium. As far as we know, this is the first report to demonstrate that physiological concentrations of AA are essential for clearance of malignant cancer cells in the presence of H₂O₂ and/or NO.

KEYWORDS | Ascorbic acid; Dehydroascorbic acid; Hydrogen peroxide; Isoascorbic acid; Nitric oxide; Reactive nitrogen species; Reactive oxygen species

ABBREVIATIONS | AA, ascorbic acid; AFR, ascorbic acid free radical; DAF-2-DA, 4,5-diaminofluorescein diacetate; DCFH-DA, 2',7'-dichlorodihydrofluorescin diacetate; DHA, dehydroascorbic acid; eNOS, endothelial nitric oxide synthase; IAA, isoascorbic acid; iNOS, inducible nitric oxide synthase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NO, nitric oxide; RNS, reactive nitrogen species; ROS, reactive oxygen species; SNP, sodium nitroprusside; SOD, superoxide dismutase

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1. INTRODUCTION

The accumulation of reactive oxygen species (ROS) and/or reactive nitrogen species (RNS) is supposedly the major mechanism involved in aging, neurodegeneration, and inflammation [1, 2]. Thus, antioxidants against ROS and/or RNS are considered to delay the pathological process of chronic diseases [3, 4]. Vitamin C (ascorbic acid, AA) has been classified as a broad-spectrum antioxidant, because it reacts with a wide array of ROS during various physiological and pathological processes [5, 6]. Thus, AA can maintain a balanced intracellular redox state and consequently protect cells from oxidative stressinduced damage. AA is involved in the first line of antioxidant defense, protecting lipid membranes and proteins from oxidative damage [5, 6].

On the other hand, as shown in **Table 1**, AA has potent toxic effects on cancer cells at higher concentrations (> 1000 μ M) [7–10]. AA selectively kills some cancer cells but not normal cells [6, 7]. As shown also in **Table 1**, the toxicity of AA itself centers on the higher concentrations (> 1000 μ M). In our previous report, we found that lower concentrations (100 μ M < [AA] < 1000 μ M) had toxic effects on non-attached cells, but not on attached ones [11], suggesting that AA may interfere with the cellular attachment, leading to potent sensitivities of cancer cells when their attachment process has not been completed. However, even these concentrations may be "unphysiologically" high because they can be only achieved by infusion of AA [12, 13]. These background data led us to consider the possibility that coordination between AA and H₂O₂/NO may clear cancer cells from the microcirculation. The physiological concentrations (< 100 μ M) of AA did not affect the cellular survival of either attached or nonattached cells. Because the concentrations of AA are strictly regulated at around 60–70 μ M in the blood in vivo [12, 13], the most important question is whether AA is protective or toxic at its physiological concentrations (< 100 μ M). Here, we found that the physiological concentrations of AA potentiated the toxic effects by H₂O₂ or NO, although AA alone did not affect cellular survival at these concentrations.

2. MATERIALS and METHODS

2.1. Chemicals

We used 3 AA derivatives [11]; they are AA, isoascorbic acid (IAA), both of which have potent reductive power, and dehydroascorbic acid (DHA), which is an oxidized product of AA and supposed to have no reductive power (**Figure 1A**). Both having the reductive "*endiol*" structure, AA and IAA can regenerate Fe²⁺, as shown in **Figure 1B**. Sodium nitroprusside (SNP), AA, DHA, IAA (sodium salt), hydrogen peroxide (H₂O₂), and 3-(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from Wako Junyaku-



TABLE 1. Concentration-dependent effects of AA on non-attached and attached COS7 cells

AA concentration	Non-attached cells	Attached cells
$[AA] > 1000 \ \mu M$	+	+
$100 \; \mu M < [AA] < 1000 \; \mu M$	+	-
[AA] <100 μM	-	-

Note: For "attached cells," the cells were incubated for 24 h before the addition of AA along with H_2O_2/NO . For "non-attached cells," AA along with H_2O_2/NO was added just after the cells had been introduced into the culture dishes. "Non-attached cells" were employed in the present study because we consider that such cells may be an in vitro model of malignant cancer cells migrating in vivo. The "+" or "–" designates "significant toxic effects" and "non-significant toxic effects," respectively. AA at > 1000 μ M could kill either non-attached cells or attached cells; at > 100 μ M, but < 1000 μ M), just non-attached cells; and at < 100 μ M did not affect cellular survival of either non-attached cells.

(Tokyo, Japan). Stock solutions of AA, DHA, and IAA (sodium salt, 100 mM or 1000 mM) were prepared in Ca²⁺, Mg²⁺-free phosphate-buffered saline (PBS, Invitrogen, Carlsbad, CA, USA). 2',7'-Dichlorodihydrofluorescin diacetate (DCFH-DA, Sigma-Aldrich, St. Louis, MO, USA) and 4,5diaminofluorescein diacetate (DAF-2-DA, Goryo Chemicals, Sapporo, Japan) stock solutions (10 mM) were prepared in dimethyl sulfoxide and used at 10 μ M in the culture medium. SNP was used as an NO donor in the present study.

2.2. Cultures of COS7 Cells

COS7 cells, which are simian malignant cancer cells, were cultured as described elsewhere [14, 15]. These cells were maintained in 10-cm dishes (Invitrogen) containing 10 ml of Dulbecco's modified Eagle medium supplemented with 10% (v/v) heat-inactivated (56°C, 30 min) fetal calf serum (Invitrogen). The cells were seeded into 24-well plates at a density of 4 x 10^4 cells/cm². When examining the toxic effects of the compounds themselves on non-attached cells, the compounds were added just after the cells had been introduced into 24-well plates.

2.3. Effects of AA on Cell Survival

We used "non-attached cells" in all experiments of the present study. The compounds (AA along with H_2O_2 or SNP) were added just after the cells had been added to 24-well plates. Then, the cells were incubated for an additional 24 h. To evaluate cell survival, we performed the MTT assay [14, 15]. In addition, "attached cells" were used (**Table 2**). In this case, the compounds (H_2O_2 or SNP) were added 24 h after the cells had been added to 24-well plates.

2.4. DCF and DAF-2 Assays

The extent of cellular oxidative stress was assessed by monitoring the formation of free-radical species by using DCFH-DA or DAF2-DA, as described elsewhere [16, 17]. Cells were plated just before initiation of the experiment at a density of 40,000 cells/well in 24-well plates. AA and 10 µM DCFH-DA or DAF2-DA were added to the cells 30 min before the measurement. The plate was set into a Spark10M apparatus (Tecan, Tokyo, Japan) under an atmosphere of 5% CO₂ and temperature of 37°C. Thereafter, 50 or 1000 µM H₂O₂ or SNP was added to the wells at 30 min. and the cells were incubated further for 180 min. DCF or DAF-2 fluorescence was measured at a 485-nm excitation wavelength and 538-nm emission wavelength at 10-min intervals. Fluorescence values were expressed as a percentage of the value obtained for the untreated control.

2.5. Statistical Analysis

Experiments presented herein were repeated at least 3 times, with each experiment performed in quadru-





FIGURE 1. Chemical structures of AA, AFR, IAA, and DHA (A) and possible involvement of regeneration of Fe^{2+} in the toxic effects (B). AA and IAA have the common chemical structure of "*endiol*" (reduced form) indicated by the dotted circle, which can give an electron(s) to other molecules (A). Proposed mechanism of preferential formation of AFR and H₂O₂ in the extracellular space (B). One of the big mysteries about AA is the cytotoxicity caused by antioxidative AA. Because catalase can prevent the AA toxic effects [6, 7], the maintenance of Fe^{2+} by a supply of electrons from AA may be the possible junction between toxic and antioxidative AA. In the extracellular space, high concentrations of AA lose 1 electron and form AFR [6, 7]. This electron reduces a protein-centered metal; an example of this reaction is shown as the reduction of Fe^{3+} to Fe^{2+} . Fe^{2+} donates an electron to oxygen, forming active oxygen including superoxide with subsequent dismutation to H₂O₂.

TABLE 2. LC50s of H₂O₂ and SNP in non-attached and attached COS7 cells

Oxidant	Non-attached cells	Attached cells
H ₂ O ₂	22.3 μM	462 μM
SNP	737 μΜ	2190 µM

Note: The "non-attached cells" and "attached cells" were described in the Materials and method section. The compounds (H_2O_2 or SNP) were added just (for "non-attached cells") or 24 h (for "attached cells") after the cells had been added to 24-well plates. Then, the cells were incubated for an additional 24 h. The MTT assay was performed to evaluate cell survival. For the MTT assay, the cells were then incubated for an additional 24 h. Values of the LC50s were calculated from the concentration-cell survival curves. LC50 denotes the concentration that kills 50% of the cells.





FIGURE 2. Potentiation of H_2O_2 toxicity by AA in non-attached COS7 cells. AA at 30 (A), 50 (B), or 70 (C) μ M was added just after the introduction of the cells into 24-well plates along with the following various concentrations (0, 20, 30, and 50 μ M) of H_2O_2 . For the MTT assay, the cells were then incubated for an additional 24 h. Open columns indicate H_2O_2 alone and gray columns show H_2O_2 +AA. Values, presented as a percentage of the control MTT value, are given as the mean \pm SD (n = 4). *, p < 0.01 compared with samples without AA.

plicate. Data were presented as the mean \pm standard deviation (SD). The statistical significance of differences was examined by performing Student's *t*-test.

3. RESULTSs

3.1. LC50s of H₂O₂ and SNP in Non-Attached and Attached Cells

H₂O₂ alone killed the non-attached COS7 cells within the range of 20–50 μ M (**Figure 2**), although AA did not kill the cells at up to 100 μ M (data not shown). The LC50 (concentration required to kill 50% of the cells) of H₂O₂ was 22.3 μ M in these nonattached cells (**Table 2**). SNP, an NO donor, killed these non-attached COS7 cells within the range of 200–1000 μ M (**Figure 3**). The LC50 of SNP was 737 μ M in these non-attached cells (**Table 2**). In contrast, these oxidants were less toxic to attached cells than to non-attached cells. LC50 of H_2O_2 and SNP was 462 μ M and 2190 μ M, respectively (**Table 2**) in the attached cells, suggesting that cellular attachment process renders these cells resistance to the lethal effects of both H_2O_2 and NO.

3.2. AA-Mediated Potentiation of H₂O₂-Induced Cell Death in Non-Attached Cancer Cells

We examined the effect of the co-presence of AA and H_2O_2 (**Figure 2**). The presence of AA at 50 μ M significantly potentiated the cell death induced by H_2O_2 although 30 μ M of AA did not affect the cell death. The potentiation by AA of H_2O_2 -induced cancer cell killing was observed within the highly narrow range of 50–70 μ M AA, suggesting that the physiological concentrations of AA could enhance the toxic effects of H_2O_2 .





FIGURE 3. AA-mediated potentiation of NO toxicity in non-attached COS7 cells. AA at 30 (A), 50 (B), or 70 (C) μ M was added just after the introduction of the cells into 24-well plates, followed by the addition of various concentrations (0, 200, 500, and 1000 μ M) of SNP. For the MTT assay, the cells were then incubated for an additional 24 h. Open columns indicate SNP alone and gray columns show SNP+AA. Values, presented as a percentage of the control MTT value, are given as the mean \pm SD (n = 4). *, p < 0.01 compared with samples without AA.

3.3. AA-Mediated Potentiation of NO-Induced Cell Death in Non-Attached Cancer Cells

Next, we examined the effect of AA and SNP added together (**Figure 3**). The presence of AA at 50 μ M significantly potentiated the cell death induced by SNP although 30 μ M of AA did not affect the cell death. Such potentiation was observed within the highly narrow range of 50–70 μ M, suggesting that the physiological concentrations of AA could enhance the toxic effects elicited by NO.

3.4. No Potentiation of H₂O₂- or NO-Induced Cell Death in Non-Attached Cancer Cells by DHA

DHA did not have any effect in terms of cellular survival of the non-attached cells at 70 μ M (**Figure 4**). The presence of DHA at 70 μ M did not at all affect the cell death induced by H₂O₂ or SNP, suggesting

that the reductive power of AA was closely linked with the potentiation of the toxic effects of H_2O_2 and NO.

3.5. Antioxidant Effects by AA against ROS and RNS in Non-Attached Cancer Cells

We compared various concentrations (30, 50, and 70 μ M) of AA in terms of levels of ROS and RNS in vitro by use of the DCF and DAF-2, respectively (**Figures 5** and **6**, respectively). AA alone at 30–70 μ M significantly reduced the baseline of ROS, whereas AA did not affect that of RNS. Because the change was very small compared with the increase in signal intensity induced by H₂O₂, the signal of the cells treated with AA alone was omitted. H₂O₂ (50 μ M) produced a significant increase (10–20-fold) in ROS in the cells. The presence of AA (50 μ M) almost abolished the increase in the levels of ROS ac-





FIGURE 4. No potentiation of H_2O_2 or NO toxicity by DHA in non-attached COS7 cells. DHA at 70 μ M was added just after the introduction of the cells into 24-well plates along with the following various concentrations (0, 20, 30, and 50 μ M) of H_2O_2 (A) or (0, 200, 500, and 1000 μ M) of SNP (B). For the MTT assay, the cells were then incubated for an additional 24 h. Open columns indicate H_2O_2 (A) or NO (B) alone and gray columns show H_2O_2 +AA (A) or NO+AA (B). Values, presented as a percentage of the control MTT value, are given as the mean \pm SD (n = 4).

tivated by 50 μ M H₂O₂, suggesting that the physiological concentrations of AA had potent antioxidative actions against H₂O₂. The ROS-scavenging effects were observed within the range of 10–100 μ M. Even 2 μ M AA significantly reduced the ROS levels increased by 50 μ M H₂O₂ (data not shown). In addition, SNP (1000 μ M), an NO donor, produced a significant increase (2–3-fold) in RNS in COS7 cells. The presence of AA (50 μ M) almost abolished the increase in the levels of RNS induced by 1000 μ M SNP, and the inhibition was observed within the range of 30–70 μ M, suggesting that the physiological concentrations of AA also had potent antioxidative actions against NO.

4. DISCUSSION

In the present study, we examined the potentiation of toxic effects of H_2O_2 or NO on non-attached cancer

cells by physiological concentrations of AA [11, 12]. The toxic effects may be closely connected with the AA reductive power because DHA, an oxidized form of AA, did not have any effect on cellular survival (Figure 4). IAA and AA share a reductive group (*endiol*), which is supposed to reduce Fe^{3+} to Fe^{2+} [6, 7]. The toxic effects are due to "endiol" chemical structures [6, 7]. AA induced selective death of cancer cells via formation of ascorbic acid free radical (AFR) and H_2O_2 in cell culture media (Figure 1B). Higher concentrations (> 1000 μ M) of AA mediated selective cancer cell toxicity via formation of AFR and H₂O₂ in cell culture media. As shown in Figure 1B, the electron lost from AA would reduce a protein-centered metal, selectively driving H2O2 formation in the extracellular fluid [6, 7]. This mechanism may work not only in the toxic effects by higher concentrations (> 1000 μ M) of AA alone, but also in the potentiation of toxic effects by the combinations of the physiological concentrations (< 100





FIGURE 5. AA-induced inhibition of ROS in non-attached COS7 cells. AA at 30 (A), 50 (B), or 70 (D) μ M along with 10 μ M DCF-DA was added just after the introduction of the cells into 24-well plates. DCF fluorescence levels are shown at 10-min intervals, and 50 μ M H₂O₂ was added at 30 min. Values are the means ± SD from 4 experiments per group. Squares, control; circles, H₂O₂; triangles, H₂O₂+AA. Note that the AA alone groups were not shown in these graphs, but AA itself slightly decreased ROS levels at all the indicated time points.

 μ M) of AA and H₂O₂ or NO. AA maintains the Fe²⁺ state by reducing Fe³⁺, which enhances the reaction. Thus, AA can kill malignant cancer cells; and the infusion of high concentrations of AA may be an alternative medicine against cancers [8–10]. AA has been reported to prevent cellular detachment from an original tissue and attachment onto distant organs during cancer metastasis [18, 19]. The supply of Fe²⁺ may be involved in the cellular attachment process [20, 21]. AA enhances the supply of Fe²⁺ to hyperactivate the enzyme proline hydroxylase, which leads to the loss of protein conformation and results in the inhibition of the cellular attachment process [22–24].

The production of H_2O_2 and/or NO by endothelial cells can be activated by the attachment of malignant

cancer cells to them, and the oxidants can kill the cancer cells in coordination with physiological concentrations of AA (**Figure 7**). This may be the defense mechanism against cancer metastasis to distal organs by AA and H_2O_2/NO at the first attachment of cancer cells onto the vascular endothelium. Because the production of H_2O_2 and NO by vascular endothelial cells is activated by the attachment of malignant cancer cells to them, these oxidants can thus kill the tumor cells in coordination with physiological concentrations AA. Thus, this coordination may be a potent mechanism for the clearance of malignant cancer cells in vivo. As shown in the **Figure 7**, cancer metastasis is initiated by the detachment of cancer cells from the original organ and their migration to distant





FIGURE 6. AA-induced inhibition of RNS in non-attached COS7 cells. AA at 30 (A), 50 (B), or 70 (D) μ M along with 10 μ M DAF-2-DA was added just after the cells had been put into 24-well plates. DAF-2 fluorescence levels are shown at 10-min intervals and 1000 μ M SNP was added at 30 min. Values are the means ± SD from 4 experiments per group. Squares, control; circles, SNP; triangles, SNP+AA. Note that the AA alone groups were not shown in these graphs, because AA itself did not affect RNS levels at any of the indicated time point.

organs, mostly via the bloodstream (1. Migration) [25, 26]. Cancer cells also express a variety of adhesion molecules that allow them to attach to the vascular endothelium (2. Adhesion), which leads to the activation of several intracellular signaling pathways (3. Signaling) [25, 26]. This adhesion of cancer cells to endothelial cells activates and induces NADPH oxidase and inducible NO synthase (iNOS), which are responsible for the production of superoxide (O_2^-) and NO, respectively [27, 28]. Because O_2^{--} is easily converted to H_2O_2 by superoxide dismutase

(SOD), the resulting H_2O_2 and AA induce toxic effects on cancer cells (Toxic effect 1). Likewise, NO kills cancer cells in the presence of AA, too (Toxic effect 2). By these events, cancer cells can be cleared from the microcirculation in distal organs.

5. CONCLUSION

In this present study, we found that physiological concentrations of AA significantly potentiated the





FIGURE 7. The AA-mediated possible clearance system of malignant cancer cells in coordination with H_2O_2/NO . Docking through adhesion molecules between cancer cells and endothelial cells can activate the NF- κ B-mediated signaling pathway leading to the induction and/or activation of NADPH oxidase and iNOS, which increases the levels of O_2 . H_2O_2 and NO in the microenvironment [25, 26]. These oxidants induce death of cancer cells in the presence of AA around 70 μ M [11, 12]. Otherwise, O_2 - and NO form ONOO⁻, which induces more potent toxic effects [29, 30]. Details are mentioned in the Discussion section.

cell death of non-attached cancer cells caused by H_2O_2 or NO, negating the increases in ROS and RNS caused by H_2O_2 or NO. Thus, the potentiation of cell death by AA must be closely linked with its reductive power. Our results thus suggest that physiological concentrations (around 70 μ M) of AA can clear malignant cancer cells from the microcirculation.

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