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COMPARISON OF ASCORBIC ACID AND ASCORBIC ACID 2-*O*-α-GLUCOSIDE ON THE CYTOTOXICITY AND BIOAVAILABILITY TO LOW DENSITY CULTURES OF FIBROBLASTS

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Abstract—Ascorbic acid 2-*O*-α-glucoside (AA-2G) is a stable ascorbate derivative which has vitamin C activity *in vivo* and *in vitro*. We studied whether AA-2G exerts a prooxidant action in cultured fibroblasts from chick embryo and human skin, as does ascorbic acid. At concentrations of 0.1–1.0 mM, ascorbic acid markedly reduced the viable cell number of low density cultures within 24 hr, whereas AA-2G had no such effect. The ascorbate cytotoxicity was dependent on the cell density at the time of its addition and it was characteristic of low density cultures. This cytotoxicity was completely prevented by catalase and partially by an Fe^{3+} ion chelator, desferrioxamine. In the early culture stage at which a morphological change in the fibroblasts began to occur, intracellular ascorbate concentrations in low density cultures after addition of ascorbic acid were much higher than in high density cultures. However, at the same concentrations, AA-2G did not cause an elevation even in low density cultures and it was also effective on collagen synthesis at high and medium densities. These results suggest that the abnormally accumulated ascorbic acid in the cells cultured at low density possibly amplifies the generation of oxygen radicals through the reduction of Fe^{3+} ions and subsequent oxidative reactions, leading to cell death. Therefore, it is concluded that AA-2G which supplies an adequate amount of ascorbic acid during culture period is a bioavailable ascorbate source without cytotoxicity.

Ascorbic acid is required by all living organisms, most importantly as an antioxidant [1] and a cofactor in hydroxylation reactions [2, 3]. It has been proved to be the most effective aqueous phase antioxidant in human blood plasma [4]. However, it can display cytotoxic effects under certain conditions. Ascorbic acid has been shown to be cytotoxic toward several types of normal and tumor cell *in vitro* [5–8]. In particular, Peterkofsky and Prather [7] have demonstrated that the growth of monolayer cultured fibroblasts derived from chick embryo and human and mouse skin was inhibited by the addition of ascorbic acid at concentrations of 0.05–0.25 mM. Jampel [8] has also described its cytotoxicity to low density cultures of Tenon's capsule fibroblasts at more than 0.5 mM. Both groups have presented a possible mechanism for the cytotoxicity of ascorbic acid to cultured fibroblasts: the generation of hydrogen peroxide during cellular oxidative reactions involving it. In fact, ascorbic acid is known to act as a prooxidant in the presence of transition metal ions [9]. However, the exact mechanism by which excess amounts of such active oxygen species are generated in the cells is not fully elucidated.

Recently, we have synthesized a novel ascorbate derivative, ascorbic acid 2-*O*-α-glucoside (AA-2G†), by regioselective enzymatic transglucosylation [10–12]. In contrast to ascorbic acid, AA-2G is characterized by its high stability under various oxidative conditions and its non-reducibility [10, 13]. AA-2G was shown to be as potent as ascorbic acid in supplementation of plasma ascorbate and antiscorbutic activity [14, 15]. In addition, we have reported its stimulatory effect on collagen synthesis in confluently cultured human skin fibroblasts and cell growth during long-term cultivation [16]. These biological activities have been found to be exerted through the slow and continuous release of ascorbic acid catalysed by α-glucosidase [16]. Furthermore, we have confirmed its *in vivo* formation in rats and guinea pigs under a specified condition [17]. These lines of evidence suggest that AA-2G is available as a predominant vitamin C both *in vitro* and *in vivo*, if it is devoid of cytotoxicity due to the prooxidant effect of ascorbic acid.

In this study, we compared the cytotoxic effect of ascorbic acid and AA-2G on cultured fibroblasts at different cell densities. We clarified that AA-2G was not cytotoxic to fibroblasts derived from chick embryo and human skin and that elevation of the intracellular concentration of ascorbic acid is possibly a trigger factor for cytotoxicity.

MATERIALS AND METHODS

Materials. The materials used in this work were obtained from the following sources: chick embryo from the Shimizu Laboratory Animals Center

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† Abbreviations: AA-2G, ascorbic acid 2-*O*-α-glucoside; MEM, minimum essential medium; PBS, phosphate-buffered saline.

(Kyoto, Japan); Eagle's minimum essential medium (MEM) from the Nissui Pharmaceutical Co. (Tokyo, Japan); sodium ascorbate and trichloroacetic acid from the Ishizu Pharmaceutical Co. (Osaka, Japan); Tricine and SDS from Wako Pure Chemical Industries (Osaka); desferrioxamine mesylate from Ciba-Geigy (Takarazuka, Japan); catalase (bovine liver, 11,000 U/mg), bovine serum albumin, α-tocopherol and dithiothreitol from the Sigma Chemical Co. (St Louis, MO, U.S.A.); bacterial collagenase (form III) from the Advance Biofactures Corp. (St Lynbrook, NY, U.S.A.); crude collagenase from Sanko Junyaku (Tokyo); fetal bovine serum from Cell Culture Technologies (Toronto, Canada); L-[2,3-^{3}H]proline from DuPont-New England Nuclear Corporation (Boston, MA, U.S.A.); trypsin solution (0.25%) from Nacalai tesque (Kyoto); neutral red from Tokyo Kasei Kogyo (Tokyo). AA-2G was synthesized as described previously [12].

Cell culture. Human abdominal dermal fibroblasts obtained from a normal child (female, 4 months old) were kindly provided by the Department of Dermatology, Shinshu University School of Medicine [18]. Cells were cultured in Eagle's MEM supplemented with 10% fetal bovine serum, 0.025 M Tricine buffer (pH 7.4) and 0.013 M $NaHCO_3$ (MEM-10) at 37° in an atmosphere of 5% CO_2–air and passaged at confluence by trypsinization (0.25%) into a culture flask (80 cm^2, Nunc). Cells from passages four through eight were used. Chick embryo fibroblasts were isolated from minced skin of 15-day-old embryos by treatment with 0.5 mg/mL crude collagenase at 37° for 30 min. Cells were cultured in MEM-10 at 37° in an atmosphere of 5% CO_2–air and passaged at confluence by trypsinization into a 80-cm^2 flask. Cells from passages three through seven were used.

Cytotoxicity assay. Different cell densities (3.3–105 × 10^3 cells/cm^2) of fibroblasts were placed in each well of a 6-well plate (9.6 cm^2/well, Nunc) or a 24-well plate (1.9 cm^2/well, Nunc). Twenty-four hours later, the medium was gently aspirated from the well and a fresh medium containing various concentrations of ascorbic acid or AA-2G was added. The ascorbate solution was prepared just before use. After 24 hr cultivation, the cells were trypsinized and microscopically counted by the dye exclusion method. In experiments using 24-well plates, the cell numbers were estimated by measuring the uptake of neutral red according to the method of Borenfreund and Puerner [19] with some modifications. In brief, 10 μL of neutral red solution (10 mg/mL) were added to each well and incubated for 1 hr. After washing the cell layer with 1 mL of phosphate-buffered saline (PBS), the cells were solubilized with 500 μL of 1% SDS at 37° for 30 min. Neutral red incorporated into the cells was fluorometrically determined in a CytoFluor fluorescent plate reader (Millipore) with an excitation at 530 nm and an emission at 645 nm. Viable cell numbers ranged from 6 × 10^3 to 2 × 10^5 cells/well and were correlated with the fluorescence intensities.

Determination of ascorbic acid. Different cell densities (3.2–13 × 10^3 cells/cm^2) of fibroblasts in MEM-10 were placed in 80-cm^2 flasks and cultured for 48 hr. The medium was changed to fresh MEM-10 containing various concentrations of ascorbic acid or AA-2G, followed by cultivation for 4 or 6 hr. After incubation, the medium was removed and the cell layer was rinsed three times with PBS. The cells were harvested by trypsinization, centrifuged and then suspended in PBS. An aliquot of the cell suspension was used to count the cell number. The remainder was centrifuged again and the cell pellet was suspended in a small volume of 5% trichloroacetic acid and centrifuged. A part of the supernatant obtained was further mixed with an equal volume of 0.05% dithiothreitol in 0.3 M potassium phosphate buffer (pH 7.0) and then incubated at 37° for 10 min. Dehydroascorbic acid was reduced to ascorbic acid by this treatment and its concentration was calculated as the difference between those of ascorbic acid and total ascorbic acid. Ascorbic acid was analysed as described previously [10] with the following modifications. The HPLC system was equipped with a pulsation damper setting between the pump and the injector, and an electrochemical detector Model ECD-100 (Eicom, Kyoto). The mobile phase was 0.1 M KH_2PO_4–H_3PO_4 buffer (pH 2.0) containing 10 mg/L EDTA with a flow rate of 0.7 mL/min. Ascorbic acid was detected at a potential +700 mV. The standard ascorbate solution was prepared at a concentration of 1 mg/mL in the HPLC buffer and freshly diluted just before use. Each result was represented as the mean of duplicate injections on HPLC.

Collagen synthesis. Different cell densities (2.6–20.8 × 10^3 cells/cm^2) of fibroblasts in MEM-10 were placed in a 6-well plate. Seventy-two hours later, the cells were incubated with 2 mL of fresh MEM-10 containing 148 kBq L-[2,3-^{3}H]proline, 0.5 mM β-aminopropionitrile fumarate and 0.25 mM AA-2G for an additional 24 hr. Collagen content in the medium was determined by the bacterial collagenase method according to our previous paper [16]. The relative rate of collagen synthesis to total protein synthesis was calculated by using a rearrangement of the formula of Peterkofsky and Diegelmann [20]. Data were represented as the means ± SD of triplicate cultures in every experiment.

Determination of protein content. Cells in a 6-well plate were washed three times with 1 mL of PBS and solubilized with 0.1 N NaOH. The protein contents were determined by the method of Lowry *et al.* [21] using bovine serum albumin as a standard.

RESULTS

Effects of ascorbic acid and AA-2G on the viability of fibroblasts

Cultures of chick embryo fibroblasts reached confluence at a cell density of around 8 × 10^4 cells/cm^2. The effects of ascorbic acid and AA-2G on the viability of fibroblasts which were cultured at a low density (1.7 × 10^4 cells/cm^2) are illustrated in Fig. 1. The addition of ascorbic acid dose-dependently decreased the viable cell number during 24 hr cultivation. At the concentration of 0.1 mM, which is close to the normal level in blood, half of the cultured cells were damaged. However, AA-2G had no effect on the viability of fibroblasts even at 1 mM, but rather tended to enhance the cell number.

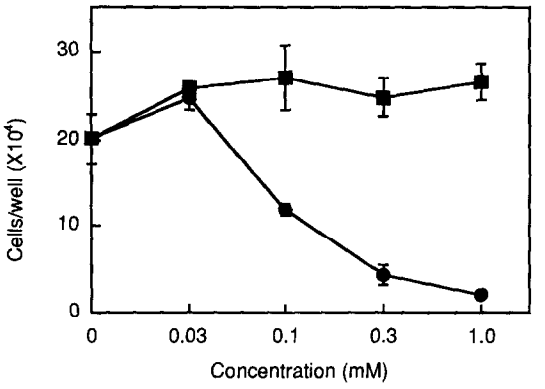

Fig. 1. Change in the viability of chick embryo fibroblasts cultured in the presence of ascorbic acid or AA-2G. Cells were seeded in a 6-well plate at 1.65×10^5 cells/well and incubated in MEM-10 for 24 hr. Cultures were maintained for an additional 24 hr in fresh MEM-10 with varying concentrations of ascorbic acid (●) or AA-2G (■). Cell numbers were microscopically counted by the dye exclusion method. Each point represents the mean ± SD of triplicate cultures.

Table 1. Toxicity of ascorbic acid and AA-2G to chick embryo fibroblasts cultured at different cell densities

Cell number at plating ($\times 10^4$ cells/well)	Cell number after incubation ($\times 10^4$ cells/well)		
	Control	Ascorbic acid (1 mM)	AA-2G (1 mM)
20	51	56 (110)	51 (100)
10	25	14 (56)	25 (100)
5.0	11	0 (0)	11 (100)
2.5	4.0	0 (0)	4.0 (100)
1.25	1.7	0 (0)	1.7 (100)

Chick embryo fibroblasts were placed in a 24-well plate (1.9 cm^2/well) at various cell densities and incubated in MEM-10 for 24 hr. Cultures were maintained for an additional 24 hr in fresh MEM-10 containing 1 mM ascorbic acid or AA-2G. Cell numbers at plating were determined by hemocytometry and those after incubation by a fluorescent assay using neutral red.

Results are the means of duplicate cultures. Figures in parentheses are percentages of the corresponding controls.

Morphological changes in chicken embryo fibroblasts cultured for an additional 6 hr in the presence of 0.3 mM ascorbic acid or AA-2G are shown in Fig. 2. Ascorbic acid did not induce any morphological change until 4 hr, but a remarkable change occurred at 6 hr. Most of the cells began to swell and separate from the culture plate (Fig. 2B) and they became completely detached and lysed. These cells were unable to be cultured again. On the contrary, AA-2G induced no change in the morphology of chick embryo fibroblasts (Fig. 2C).

Effect of ascorbic acid and AA-2G on cell density-dependent cytotoxicity

Ascorbate toxicity was further examined in relation to the cell density of fibroblasts. As shown in Tables 1 and 2, the cytotoxicity of 1 mM ascorbic acid was dependent on the cell density of fibroblasts from both chick embryo and human skin. Although chick embryo fibroblasts at confluent growth (initial density: 1.05×10^5 cells/cm^2) were completely resistant to this toxicity, low density cultures of less than 5×10^4 cells/cm^2 at plating time were damaged by 1 mM ascorbic acid. Similarly, human skin fibroblasts, which are considerably larger in size than chick

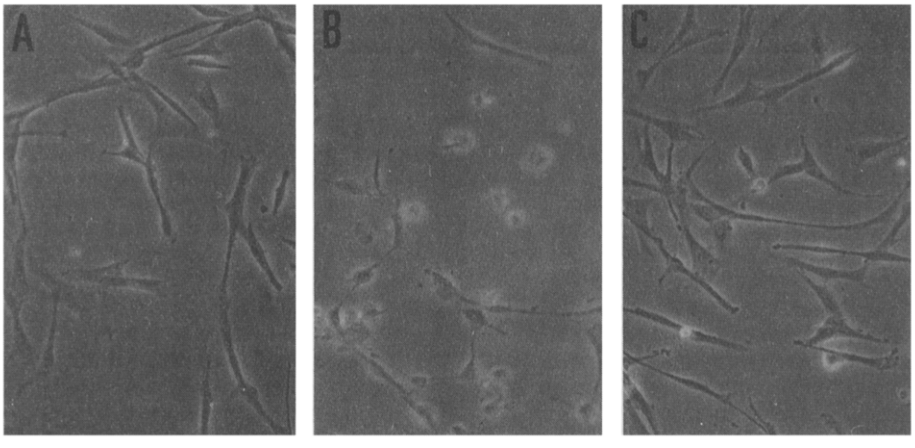

Fig. 2. Morphological change in chick embryo fibroblasts cultured in the presence of ascorbic acid or AA-2G. Cells were seeded in a 6-well plate at 1.0×10^5 cells/well and incubated in MEM-10 for 24 hr. Cultures were maintained for an additional 6 hr in fresh MEM-10 containing 0.3 mM ascorbic acid (B) or 0.3 mM AA-2G (C). Control cells are shown in (A). After incubation, representative fields were photographed.

Table 2. Toxicity of ascorbic acid and AA-2G to human skin fibroblasts cultured at different cell densities

Cell number at plating ($\times 10^4$ cells/well)	Cell number after incubation ($\times 10^4$ cells/well)		
	Control	Ascorbic acid (1 mM)	AA-2G (1 mM)
10	6.6	4.4 (66)	9.6 (145)
5.0	3.1	0.2 (6)	3.4 (110)
2.5	1.4	0 (0)	1.4 (100)
1.25	0.80	0 (0)	0.74 (93)
0.625	0.35	0 (0)	0.37 (106)

Experimental conditions were the same as in Table 1 except that human skin fibroblasts were used.

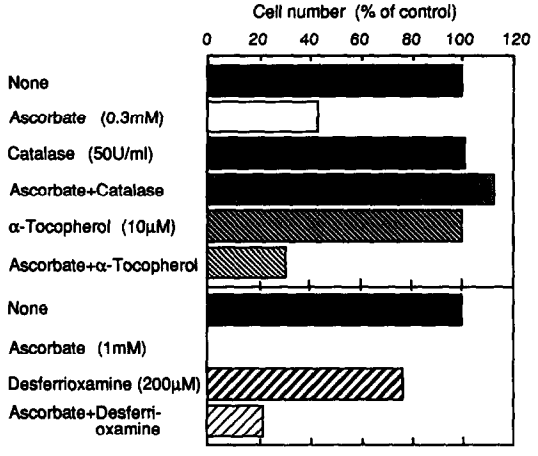

Fig. 3. Effect of catalase, α-tocopherol and desferrioxamine on ascorbate cytotoxicity to chick embryo fibroblasts. Cells were placed in a 24-well plate at 7.0×10^4 cells/well and incubated in MEM-10 for 24 hr. Cultures were maintained for an additional 24 hr in fresh MEM-10 containing the compounds listed. Cell numbers were determined by the fluorescent assay using neutral red. Results are the means of duplicate cultures and representative of three separate experiments.

embryo cells, were damaged in low density cultures. However, the same concentration of AA-2G showed no cytotoxicity to any cell density cultures of either type of fibroblast.

Participation of active oxygen in ascorbate cytotoxicity

The effects of catalase, α-tocopherol and desferrioxamine on ascorbate toxicity to chick embryo fibroblasts were next determined. As shown in Fig. 3, the addition of catalase (50 U/mL) to the medium completely prevented the cytotoxicity induced by 0.3 mM ascorbic acid, whereas α-tocopherol (10 μM) showed no preventive effect. Desferrioxamine, a potent chelator of Fe^{3+} but not Fe^{2+} ions [22], partly prevented the ascorbate toxicity at 200 μM, although itself possesses a weak cell-damaging effect. This result indicated that Fe^{3+} ions are also involved in this oxidative cell damage.

Intracellular concentration of ascorbic acid

To clarify the difference between ascorbic acid and AA-2G in cytotoxicity to cultured fibroblasts, ascorbate concentrations in the cells cultured with either compound were determined by HPLC. The detectable limit of ascorbic acid in this assay method was 10 fmol and chick embryo fibroblasts cultured in the absence of these compounds contained indetectable amounts of ascorbic acid. When the low density cultures of chick embryo fibroblasts were incubated with 0.25 mM ascorbic acid, the intracellular levels of ascorbic acid increased rapidly with the culture period, reaching a maximum at 6 hr (data not shown). This coincided with the time at which ascorbate cytotoxicity began to appear. Therefore, initial changes occurring in the cells at 4 and 6 hr after the addition of 0.25 mM ascorbic acid were estimated by employing different cell density cultures of chick embryo fibroblasts. The results are summarized in Table 3. The intracellular concentrations of total ascorbic acid were in reverse relation to the cell density. The uptake of ascorbic acid per cell was much higher in low density cultures than in high density cultures. In every culture, dehydroascorbic acid was detected in relatively higher amounts than its reduced form. Table 4 demonstrates the difference between intracellular ascorbate concentrations in the low density cultures of fibroblasts incubated with 1 mM ascorbic acid and AA-2G for 4 hr. AA-2G-treated cells incorporated only one-twelfth of the amount of ascorbic acid incorporated by ascorbate-treated cells.

Effect of AA-2G on collagen synthesis by low density cultures of fibroblasts

In our previous paper [16], AA-2G was found to have a potent stimulatory effect on collagen synthesis in confluent cultures of human skin fibroblasts. We determined further whether AA-2G possesses the ability to stimulate collagen synthesis in low density cultures. As summarized in Table 5, 0.25 mM AA-2G exerted its stimulatory activity at the initial cell density of more than 5×10^4 cells/9.6 cm^2, though the rates of collagen synthesis were considerably lower in low density than in high density cultures.

DISCUSSION

In the present study, we demonstrated that ascorbic acid exhibited a cytotoxic effect on human skin and chick embryo fibroblasts under low density culture conditions and that AA-2G, a stable ascorbate derivative, showed no cytotoxicity to these cells. Although the cytotoxicity of ascorbic acid has been considered to be mediated via its own prooxidant action, such as the generation of hydrogen peroxide [7, 8, 23], a detailed mechanism has not yet been substantiated. By comparing the difference between ascorbic acid and AA-2G in the cytotoxicity and bioavailability to fibroblasts, we obtained the following findings suggesting that the striking elevation of ascorbic acid in the cell should amplify subsequent oxidative reactions. First, a

Table 3. Intracellular ascorbate concentrations in chick embryo fibroblasts cultured at different cell densities in 0.25 mM ascorbate-containing medium

Incubation time (hr)	Cell density at harvest (cells/cm^2)	Intracellular concentration (pmol/10^6 cells)		
		Total ascorbate	Ascorbate	Dehydro-ascorbate
4	2.45×10^4	14.9	5.2	9.7
	1.28×10^4	25.8	8.6	17.2
6	7.26×10^4	13.8	7.2	6.6
	3.16×10^4	17.0	6.5	10.5
	1.53×10^4	44.3	19.1	25.2

Cells were placed in a 80-cm^2 flask at different cell densities and cultured for 48 hr. Cultures were maintained in fresh MEM-10 containing 0.25 mM ascorbic acid for an additional 4 or 6 hr. After incubation, cells were harvested and cell numbers were counted. Intracellular ascorbate concentrations were determined by HPLC.

Results are the means of duplicate assays.

Table 4. Comparison of intracellular ascorbate concentrations in chick embryo fibroblasts cultured at low density in 1 mM ascorbate- or AA-2G-containing medium

Compound	Intracellular concentration (pmol/10^6 cells)		
	Total ascorbate	Ascorbate	Dehydro-ascorbate
Ascorbate acid	69.5	34.4	35.1
AA-2G	5.8	2.4	3.4

Cells were placed in a 80-cm^2 flask at low density and cultured for 48 hr. Cultures were maintained in fresh MEM-10 containing 1 mM ascorbic acid or AA-2G for an additional 4 hr. After incubation, cells were harvested and a cell density of around 2.5×10^4 cells/cm^2 was determined. Intracellular ascorbate concentrations were determined by HPLC.

Results are the means of duplicate assays.

morphological change in cultured fibroblasts caused by ascorbic acid was observed 6 hr after its addition and intracellular levels of ascorbic acid also reached a maximum at this time. Second, cytotoxicity induced by ascorbic acid was dependent on the cell density at the time of addition of ascorbic acid and it increased with reduction in cell density. The uptake of ascorbic acid into the cell was much higher in low density than in high density cultures. Third, ascorbate-treated fibroblasts incorporated ascorbic acid to a much greater degree than AA-2G-treated cells. Thus, these lines of evidence support the relationship between ascorbate toxicity and its intracellular concentration in cultured cells.

The ascorbate cytotoxicity to both types of cultured fibroblast was completely abolished by the addition of catalase, a scavenger of hydrogen peroxide, but not by a lipophilic antioxidant α-tocopherol. The participation of hydrogen peroxide in this cytotoxicity has already been described by other researchers [7, 8, 23] but it is difficult to explain ascorbate cytotoxicity by hydrogen peroxide only. Des-

Table 5. Effect of AA-2G on collagen synthesis in human skin fibroblasts cultured at different cell densities

Cell density at plating (cells/well)	Compound	Cell protein (μg/well)	Relative rate of collagen synthesis (%)
20×10^4	None	194	8.44 ± 0.59
	AA-2G	216	15.52 ± 1.78
10×10^4	None	165	8.90 ± 0.81
	AA-2G	167	17.21 ± 0.76
5×10^4	None	123	6.72 ± 0.03
	AA-2G	143	10.60 ± 1.51
2.5×10^4	None	81	7.85 ± 0.91
	AA-2G	91	8.69 ± 0.96

Human skin fibroblasts were placed in a 6-well plate (9.6 cm^2/well) at different cell densities and cultured for 72 hr. Cultures were maintained for an additional 24 hr in fresh MEM-10 in the presence or absence of 0.25 mM AA-2G. After incubation, the cell layer was subjected to the determination of cellular protein and the medium of rate of collagen synthesis relative to total protein synthesis.

The latter results are represented as the means ± SD of triplicate cultures.

ferrioxamine, an Fe^{3+} ion chelator, could prevent ascorbate cytotoxicity in part, suggesting the participation of Fe^{3+} ions in this oxidative damage. Fe^{3+} ions are effectively reduced to Fe^{2+} ions in the presence of ascorbic acid and, moreover, ascorbic acid has been described as having the ability to release Fe^{2+} ions from ferritin [24]. Free Fe^{2+} ions thus formed can mediate the generation of more active free radical species such as hydroxy radicals from hydrogen peroxide through the Fenton reaction [25]. Hydroxy radicals react indiscriminately with the closest neighboring molecules, such as chromosomal DNA and membrane components, leading to cell death. It has been reported that DNA fragmentation occurred in human fibroblasts cultured with ascorbic acid and Cu^{2+} ions [26–28]. In addition, ascorbate cytotoxicity was also associated with membrane damage, because the uptake of neutral red, a marker event of membrane integrity [19], was diminished. The present results suggest that excess amounts of ascorbic acid accumulated in the low density cells probably amplify the production of free radicals in large quantities. This is supported by the observation by Murata *et al.* [29] that the *in vitro* bactericidal action of ascorbic acid in the presence of a trace level of Cu^{2+} ions is mediated by hydroxy radicals generated at the inner side of the cell membrane. However, the final event in this cytotoxic reaction remains to be investigated.

It is also possible to speculate that extracellular ascorbic acid partly exhibits cytotoxicity by the mechanism of free radical generation. Ascorbic acid is readily oxidized in every culture medium containing low concentrations of serum [13, 16]. In addition, the susceptibility of cell membranes to cell damaging radicals may be influenced by the stage of cell density-induced proliferation. Therefore, this consideration suggests disadvantages of ascorbic acid for *in vitro* use directed toward the elucidation of its physiological functions. In contrast, AA-2G was shown to be not only non-cytotoxic at all concentrations used but also effective on collagen synthesis by high and medium density cultures of fibroblasts. This presumably arises from its slow and continuous hydrolysis by cellular α-glucosidase [16] and subsequent specific transport mechanism [30]. Therefore, a trace amount of AA-2G should be hydrolysed by α-glucosidase, and ascorbic acid released selectively transported into the cell to express its full activities. As ascorbic acid is known to be transported into several kinds of cells with saturable kinetics [31, 32], its overdosing in every *in vitro* culture system is not necessarily recommended. Since the use of a high level of ascorbic acid resulted in its instability in neutral aqueous solution [13, 16], stable derivatives such as AA-2G and ascorbic acid 2-phosphate [33] should be used for the *in vitro* study in the place of ascorbic acid. These two derivatives are evaluated to have the same potential as an ascorbate source [14, 16], although AA-2G is superior to the phosphate ester in terms of synthetic efficiency.

In general, both passaged cell lines and primary cultured cells which have been incubated in an ascorbate-free medium are in an ascorbate-deficient state. It is therefore of great importance that the elucidation of normal cell functions should be carried out *in vitro* by using the medium supplemented with physiological concentrations of ascorbic acid. We have recently found that AA-2G showed a marked enhancing effect on antibody production in 5-day cultured mouse splenocytes (manuscript in preparation). This effect was not found by a single addition of ascorbic acid, but observed by its repeated addition, although the repeated addition of 0.5 mM ascorbic acid every 12 hr resulted in a drop in lymphocyte viability. Furthermore, we have demonstrated that AA-2G was more effective than ascorbic acid in the prevention of hydrocortisone-induced cataract formation in developing chick embryos [34]. This difference in their potencies may also be dependent on direct cytotoxicity, though the mechanism remains to be elucidated. Taken together, it is concluded that AA-2G is a very useful ascorbate derivative in terms of its stability, bioavailability and non-cytotoxicity. Its application to various multicomponent liquid products such as medicines, foods and cosmetics, as well as to *in vitro* culture systems is widely expected.

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