

## Collagen Synthesis in Human Skin Fibroblasts is Stimulated by a Stable Form of Ascorbate, 2-O- $\alpha$ -D-Glucopyranosyl-L-Ascorbic Acid<sup>1</sup>

ITARU YAMAMOTO,<sup>2</sup> NORIO MJTO, KOUKI MURAKAMI  
AND JUN-ICHI AKIYAMA

Department of Immunochemistry, Faculty of Pharmaceutical Sciences,  
Okayama University, Tsushima-naka 1-1-1, Okayama 700, Japan

**ABSTRACT** We evaluated the effect of 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G) on collagen synthesis in cultured human skin fibroblasts and on proliferation of fibroblasts. At concentrations of 0.1–0.5 mmol/L, AA-2G effectively stimulated collagen synthesis with an effectiveness comparable to that of L-ascorbic acid. On the other hand, 6-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid showed a weak effect. The stimulation of collagen synthesis by AA-2G was attenuated by the addition of a collagen synthesis inhibitor, L-azetidine 2-carboxylic acid, in a dose-dependent manner. In addition, AA-2G-induced stimulation of collagen synthesis could be completely inhibited by the addition of castanospermine, an inhibitor of neutral  $\alpha$ -glucosidase. Relatively high  $\alpha$ -glucosidase activity, which would contribute to release of ascorbic acid from AA-2G, could be detected in the lysate of cultured fibroblasts. The stimulatory activity of AA-2G on collagen synthesis was observed after 5 d in culture, whereas L-ascorbic acid tended to lose its stimulatory activity. Continuous supplementation of AA-2G (0.25 mmol/L) to culture medium for 24 d enhanced the cell growth four times that of the control. These results indicate that AA-2G is gradually cleaved by the cellular  $\alpha$ -glucosidase to release L-ascorbic acid, which adequately stimulates collagen synthesis and proliferation of human skin fibroblasts. *J. Nutr.* 122: 871–877, 1992.

**INDEXING KEY WORDS:**

- vitamin C • ascorbic acid 2-glucoside
- ascorbic acid • collagen synthesis
- human skin fibroblasts

L-Ascorbic acid functions in many biological processes such as collagen synthesis, anti-oxidation, intestinal absorption of iron and metabolism of some amino acids (1). An essential function of L-ascorbic acid is to act as a cofactor for the hydroxylation of proline and lysine residues in collagen, a major protein component of the body. L-Ascorbic acid also

increases transcription rate of procollagen genes and stabilizes procollagen mRNA (2, 3). Its ability to cure scurvy is possibly due to the stimulation of collagen synthesis in connective tissues (4). L-Ascorbic acid is unstable in aqueous solutions, even under normal culture conditions at neutral pH and 37°C. To solve this problem, a number of stable derivatives including L-ascorbic acid 2-sulfate (AA-2S)<sup>3</sup> and L-ascorbic acid 2-phosphate (AA-2P), have been developed (5, 6). L-Ascorbic acid 2-phosphate shows antiscorbutic activity in guinea pigs (7, 8) and stimulatory activity of collagen synthesis in cultured human skin fibroblasts (9, 10), but AA-2S is not effective in this way (11, 12).

In 1971, Suzuki et al. (13) demonstrated the formation of an L-ascorbic acid glucoside with  $\alpha$ -glucosidase from *Aspergillus niger*, with the chemical structure 6-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-6G) assigned by them in 1989 (14). Recently, we have found a novel L-ascorbic acid glucoside, 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G, Fig. 1) (15), which is formed enzymatically with mammalian  $\alpha$ -glucosidase (16, 17), rice seed  $\alpha$ -glucosidase (18) or *Bacillus stearothermophilus* cyclodextrin glucanotransferase (19, 20) by regio-specific transglucosylation. This ascorbate derivative, in contrast to L-ascorbic acid and AA-6G, is characterized by its high stability in various aqueous solutions throughout wide ranges of pH and its nonreducibility (15). The

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<sup>2</sup>To whom correspondence should be addressed.

<sup>3</sup>Abbreviations used: AA-2G, 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid; AA-6G, 6-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid; AA-2P, L-ascorbic acid 2-phosphate; AA-2S, L-ascorbic acid 2-sulfate; AzC, L-azetidine 2-carboxylic acid; MEM, minimum essential medium; MEM-10, minimum essential medium with 10% fetal bovine serum.

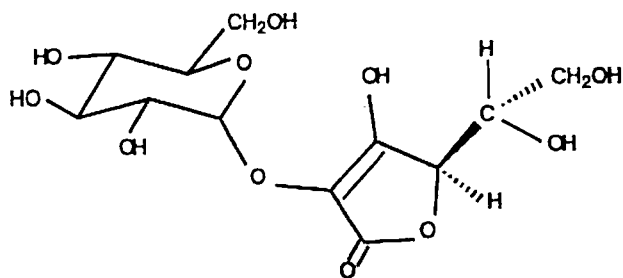


FIGURE 1 Chemical structure of L-ascorbic acid 2-O- $\alpha$ -D-glucoside.

ascorbate moiety in this glucoside is fully protected from oxidation if the bond is not cleaved. However, this glucoside has been demonstrated not only to serve as an L-ascorbic acid supplement when orally or intravenously administered to experimental animals, but also to exhibit a potent therapeutic activity in scorbutic guinea pigs [21] and to exhibit antiscorbutic activity in guinea pigs fed a vitamin C-deficient diet (unpublished data). These findings mean that L-ascorbic acid, released from AA-2G by enzymatic hydrolysis, exhibits these biological activities.

In this work, we showed that AA-2G effectively stimulated collagen synthesis in cultured human skin fibroblasts and also enhanced cell growth in long-term cultivation. We considered that this activity might be expressed by specific and mild hydrolysis catalyzed by  $\alpha$ -glucosidase.

## MATERIALS AND METHODS

**Chemicals.** The sources of materials used in this work were as follows: Eagle's minimum essential medium (MEM) from Nissui Pharmaceutical, Tokyo, Japan; AA-2G and AA-6G from Hayashibara Biochemical Labs, Okayama, Japan; sodium salt of L-ascorbic acid from Ishizu Pharmaceutical, Osaka, Japan; magnesium salt of AA-2P from Wako Pure Chemical Industries Ltd., Osaka, Japan; potassium salt of AA-2S and L-azetidine 2-carboxylic acid (AzC) from Sigma Chemical, St. Louis, MO; castanospermine from Boehringer Mannheim Yamanouchi, Tokyo, Japan; collagenase form III from Advance Biofacture, St. Lynbrook, NY; L-[2,3- $^3$ H]proline from New England Nuclear, E. I. Dupont, Boston, MA.

**Cell culture.** Human forearm skin fibroblasts from a normal child (female, 2-y-old) were kindly provided from Department of Dermatology, Shinshu University, School of Medicine and used at population doubling levels of 15–30. Cells ( $2\text{--}5 \times 10^4$ ) were plated onto 35-mm dishes and grown in Eagle's MEM supplemented with 10% fetal bovine serum (MEM-10) at

37°C for 10–14 d in an atmosphere of 5% CO<sub>2</sub>-air until apparent confluence was obtained.

**Determination of L-ascorbic acid and 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid in culture medium.** L-Ascorbic acid and AA-2G were dissolved in distilled water at a concentration of 25 mmol/L, and sterilized by filtration. Each solution was added to MEM-10 at a final concentration of 0.25 mmol/L in the absence or presence of fibroblasts and then incubated at 37°C for 60 h in an atmosphere of 5% CO<sub>2</sub>-air. L-Ascorbic acid and AA-2G in culture medium were measured by HPLC as described previously [16].

**Collagen synthesis.** Collagen synthesis was determined by measuring labeled proline in collagen and noncollagenous protein by using purified collagenase [22]. The cells were incubated with 2.0 mL of fresh MEM-10 containing L-[2,3- $^3$ H]proline (148 kBq/dish), 0.5 mmol/L  $\beta$ -aminopropionitrile fumarate and varying amounts of L-ascorbic acid-related compounds and cultured for 24–120 h. In some experiments, this cultivation was carried out in the presence of AzC (0.5 and 1.0 mmol/L) or castanospermine (0.1 mmol/L). The medium was collected and then mixed with 1 mL of 1.84 mol/L trichloroacetic acid to precipitate high-molecular-mass proteins. The precipitate obtained was dissolved in 1 mL of 0.1 mol/L Tris-HCl buffer (pH 7.6) and added with chilled ethanol at a final concentration of 66%. The precipitate was dried in vacuo and the sample was dissolved in 300  $\mu$ L of 0.2 mol/L NaOH, followed by neutralization with 0.3 mol/L HCl to give a pH of 6–8. The samples were subjected to collagenase digestion. Reaction mixtures consisting of 100  $\mu$ L of sample, 10  $\mu$ L of collagenase solution (5 units), 10  $\mu$ L of 25 mmol/L CaCl<sub>2</sub>, and 20  $\mu$ L of 62.5 mmol/L N-ethylmaleimide were incubated at 37°C for 90 min and mixed with 250  $\mu$ L of 0.61 mol/L trichloroacetic acid-5 g/L tannic acid. The precipitate was treated again with 500  $\mu$ L of 0.31 mol/L trichloroacetic acid-2.5 g/L tannic acid. Acid-soluble fractions were combined and acid-insoluble materials were solubilized with 500  $\mu$ L of 0.1 mol/L Tris-HCl buffer (pH 7.6) containing 17.3 mmol/L sodium dodecyl sulfate and 5 mmol/L dithiothreitol at 95°C for 5 min. Both fractions were estimated as collagen and noncollagen proteins, respectively, and their radioactivities were counted in a LKB scintillation counter (LKB-Produkter AB, Bromma, Sweden). The relative rate of collagen synthesis to total protein synthesis was calculated by using a rearrangement of the formula of Diegelmann and Peterkofsky [23]: relative rate of collagen synthesis to total protein synthesis (%) = [Bq of collagen/[Bq of collagen + (5.4  $\times$  Bq of noncollagen)]]  $\times$  100.

**Quantification of DNA content.** Cells ( $5 \times 10^4$ ) were plated onto 35-mm dishes and grown in MEM-10. After 24 h, the medium was changed to fresh MEM-10 or fresh MEM-10 containing 0.25 mmol/L

AA-2G, and the cells were cultured for various times until 24 d. The medium was changed twice weekly. After removal of the medium, the cell layer was rinsed twice with 2 mL of PBS (pH 7.2) and processed for the measurement of DNA content as described by Giles and Myer (24).

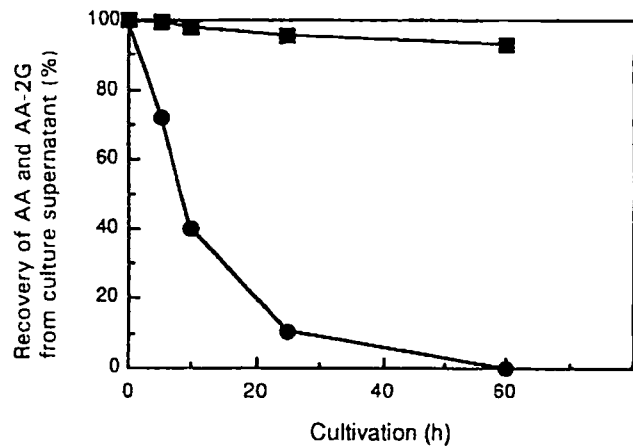
#### Determination of enzyme activity of cell lysate.

The cell suspension ( $4 \times 10^9$  cells/L) in 0.1 mol/L barbiturate buffer (pH 7.2) was sonicated and centrifuged at  $1700 \times g$  for 10 min. The supernatant was used for the determination of various enzyme activities at 25°C and at optimal pH.  $\alpha$ -Glucosidase (EC 3.2.1.20) activity was determined by measuring the rate of formation of glucose from maltose as described previously (17). Alkaline phosphatase (EC 3.1.3.1) activity was measured with 7.05 mmol/L *p*-nitrophenyl phosphate in 0.72 mol/L diethanolamine-HCl buffer (pH 9.8). Arylsulfatase (EC 3.1.6.1) activity was measured with 1.81 mmol/L *p*-nitrophenyl sulfate in 87 mmol/L acetate buffer (pH 6.2). *p*-Nitrophenol formed in these reactions was measured at 405 nm. L-Ascorbic acid-liberating activities of the cell lysate were also determined at pH 7.2 with AA-2G, AA-2P or AA-2S. The reaction mixture consisting of 30  $\mu$ L of enzyme solution and 10  $\mu$ L of 50 mmol/L L-ascorbic acid derivative was incubated at 37°C for 20 min, followed by addition of 2 volumes of 0.61 mol/L trichloroacetic acid. To the reaction mixture was added an equal volume of 52 mmol/L dithiothreitol in 1 mol/L potassium phosphate buffer (pH 7.0). The sample obtained was analyzed for total L-ascorbic acid amount by HPLC (16) equipped with an Eicom electrochemical detector. Protein concentration was determined by the method of Lowry et al. (25) using bovine serum albumin as a standard. Specific activity of each enzyme was expressed as nmol of substrate hydrolyzed/(min·mg protein).

**Statistical analysis.** Means and standard deviations are presented and were compared by Student's *t* test with significant probability levels of  $<0.05$ .

## RESULTS

Because L-ascorbic acid is unstable in aqueous solution, the stability of AA-2G in our culture medium was first determined. Figure 2 illustrates the rate of disappearance of L-ascorbic acid and AA-2G from the culture medium of fibroblasts. L-Ascorbic acid disappeared rapidly during the culture period and  $<10\%$  remained after 25-h incubation. In contrast, AA-2G was quite stable under the culture conditions and  $>90\%$  of the intact form remained even after 60-h incubation. The similar stability of AA-2G was observed in MEM-10 without cells. Under this culture condition, AA-2G could not be detected in fibroblast cells by our HPLC method.



**FIGURE 2** Rate of disappearance of L-ascorbic acid (AA) and 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G) in culture medium. AA or AA-2G was added to MEM-10 (minimum essential medium with 10% fetal bovine serum) at a final concentration of 0.25 mmol/L in the presence of fibroblasts and then incubated at 37°C for 60 h in an atmosphere of 5% CO<sub>2</sub>-air. The remaining amounts of AA (•) and AA-2G (■) in the medium were measured by HPLC. Points are the means of quadruplicate cultures.

The effects of AA-2G and other L-ascorbic acid derivatives on collagen synthesis by cultured fibroblasts are summarized in Table 1. AA-2G significantly stimulated collagen synthesis as well as L-ascorbic acid and AA-2P did, whereas AA-6G and AA-2S showed weak or no enhancing effects. Among

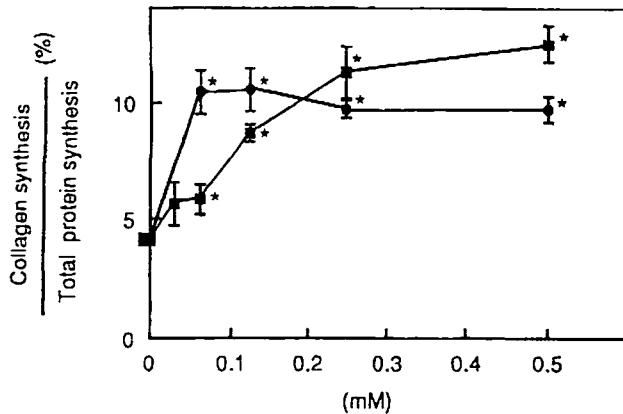
**TABLE 1**

*Effect of concentration of L-ascorbic acid (AA) derivatives on collagen synthesis as a percentage of total protein synthesis in human skin fibroblasts<sup>1</sup>*

Stimulator	Concentration	
	0.1 mmol/L	0.25 mmol/L
	%	
None	3.3 $\pm$ 0.3	3.3 $\pm$ 0.3
AA	11.0 $\pm$ 0.4*	10.1 $\pm$ 0.7*
AA-2G <sup>2</sup>	8.6 $\pm$ 0.9*	9.0 $\pm$ 0.6*
AA-6G	6.3 $\pm$ 0.4*	6.2 $\pm$ 0.3*
AA-2P	9.1 $\pm$ 0.4*	8.0 $\pm$ 0.5*
AA-2S	3.4 $\pm$ 0.2	4.8 $\pm$ 0.4*

<sup>1</sup>Cells were incubated in 2.0 mL of fresh MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [<sup>3</sup>H]proline (74 MBq/L) and AA derivatives (0.1 and 0.25 mmol/L) for 24 h. The relative rate of collagen synthesis to total protein synthesis was determined by the bacterial collagenase method. Values are the means  $\pm$  SD of triplicate cultures. \*Significantly different compared with corresponding control (none) ( $P < 0.05$ ).

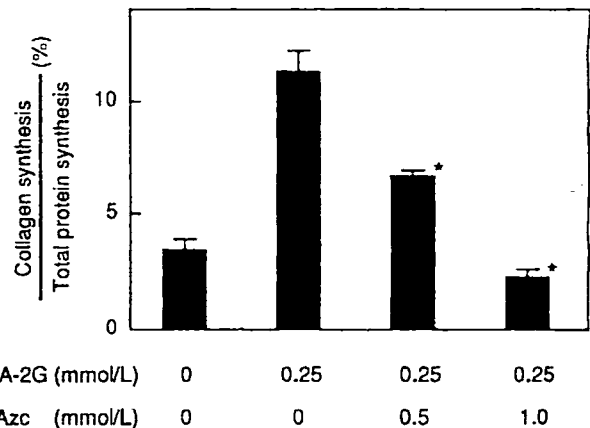
<sup>2</sup>Abbreviations used: AA-2G, 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid; AA-6G, 6-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid; AA-2P, L-ascorbic acid 2-phosphate; AA-2S, L-ascorbic acid 2-sulfate.



**FIGURE 3** Effect of increasing concentrations of L-ascorbic acid (AA) and 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G) on collagen synthesis in human skin fibroblasts. The cells were incubated in 2.0 mL of fresh MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [ $^3$ H]proline (74 MBq/L) and AA ( $\bullet$ ) or AA-2G ( $\blacksquare$ ) (0.03–0.5 mmol/L) for 24 h. The relative rate of collagen synthesis to total protein synthesis was determined by the bacterial collagenase method. Points are the means  $\pm$  SD of triplicate cultures. \*Significantly different than value determined in the absence of AA or AA-2G ( $P < 0.05$ ).

these L-ascorbic acid-related compounds, L-ascorbic acid and AA-6G showed a reducing activity toward cytochrome *c* and a redox dye, whereas AA-2G, AA-2P, and AA-2S had no direct reducing activity. Consequently, there was no relationship between their stimulating effect on collagen synthesis and their direct reducibility. As shown in Figure 3, the stimulation of collagen synthesis by AA-2G was dose-dependent at concentrations ranging from 0.03 to 0.5 mmol/L. However, L-ascorbic acid strongly stimulated collagen synthesis at a concentration as low as 0.06 mmol/L and reached a maximum at 0.125 mmol/L. The reason for this relatively weak effect of L-ascorbic acid at concentrations more than 0.25 mmol/L on collagen synthesis is not clear, but this may be due to cytotoxicity of hydrogen peroxide produced by oxidative reaction of L-ascorbic acid under culture conditions (26). On the contrary, a high concentration of AA-2G (2.0 mmol/L) was as effective as 0.25 mmol/L in stimulating collagen synthesis (data not shown).

Figure 4 illustrates the effect of AzC on AA-2G-induced collagen synthesis. L-Azetidine 2-carboxylic acid, an inhibitor of collagen synthesis induced by L-ascorbic acid (27), attenuated the stimulatory effect of AA-2G on collagen synthesis in a dose-dependent manner. We further determined the effect of castanospermine, a neutral  $\alpha$ -glucosidase inhibitor (28), on AA-2G-induced collagen synthesis. As shown in Table 2, this compound completely inhibited AA-2G-induced collagen synthesis, but not that induced by L-ascorbic acid. Table 3 shows the



**FIGURE 4** Effect of azetidine 2-carboxylic acid (AzC) on 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G)-stimulated collagen synthesis in human skin fibroblasts. The cells were incubated in 2.0 mL of fresh MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [ $^3$ H]proline (74 MBq/L), AA-2G (0.25 mmol/L) and AzC for 24 h. The relative rate of collagen synthesis to total protein synthesis was determined by the bacterial collagenase method. Points are the means  $\pm$  SD of triplicate cultures. \*Significantly different than value from cells stimulated by AA-2G only ( $P < 0.05$ ).

three enzyme activities in the lysate of cultured fibroblasts. The cell lysate exhibited  $\alpha$ -glucosidase activity and had the ability to release L-ascorbic acid from AA-2G. These values were relatively higher than the other two enzyme activities examined. Among the three L-ascorbic acid derivatives, AA-2S was the most

**TABLE 2**

*Abolishment of 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G)-induced collagen synthesis in human skin fibroblasts by castanospermine<sup>1</sup>*

Stimulator	Castanospermine	Collagen synthesis
		% total protein synthesis
None	-	5.2 $\pm$ 0.7
None	+	5.7 $\pm$ 0.5
AA-2G	-	8.6 $\pm$ 0.3*
AA-2G	+	5.1 $\pm$ 0.3
L-Ascorbic acid	-	13.0 $\pm$ 0.8*
L-Ascorbic acid	+	13.1 $\pm$ 1.2*

<sup>1</sup>Cells were incubated in 2.0 mL of fresh MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [ $^3$ H]proline (74 MBq/L) and L-ascorbic acid or AA-2G (0.1 mmol/L) in the presence or absence of castanospermine (0.1 mmol/L) for 24 h. The relative rate of collagen synthesis to total protein synthesis was determined by the bacterial collagenase method. Values are the means  $\pm$  SD of triplicate cultures. \*Significantly different compared with control value (none) in the absence of castanospermine ( $P < 0.05$ ).

TABLE 3  
Enzyme activities of cultured fibroblasts<sup>1</sup>

Enzyme	pH	Substrate	Specific activity nmol hydrolyzed/(min·mg protein)
α-Glucosidase	6.0	Maltose	3.70
Alkaline phosphatase	9.8	<i>p</i> -Nitrophenyl phosphate	2.41
Arylsulfatase	6.2	<i>p</i> -Nitrophenyl sulfate	0.40
AA-releasing activity <sup>2</sup>	7.2	AA-2G	11.1
	7.2	AA-2P	1.16
	7.2	AA-2S	0.013

<sup>1</sup>Cell suspension ( $4 \times 10^9$  cells/L) was lysed and the lysate was used as an enzyme source. Neutral α-glucosidase, alkaline phosphatase and arylsulfatase activities toward maltose, *p*-nitrophenyl phosphate and *p*-nitrophenyl sulfate, respectively, were measured at 25°C and at individual optimal pH. L-Ascorbic acid-releasing activities toward 2-*O*-α-D-glucopyranosyl-L-ascorbic acid, L-ascorbic acid 2-phosphate and L-ascorbic acid 2-sulfate were determined at 37°C and pH 7.2. Values are the means of triplicate assays from cell lysate.

<sup>2</sup>Abbreviations used: AA, L-ascorbic acid; AA-2G, 2-*O*-α-D-glucopyranosyl-L-ascorbic acid; AA-2P, L-ascorbic acid 2-phosphate; AA-2S, L-ascorbic acid 2-sulfate.

inferior L-ascorbic acid source, supporting its extremely low activity in collagen synthesis.

We further compared the durability of stimulatory effects of L-ascorbic acid and AA-2G on collagen synthesis during 5-d cultivation (Fig. 5). AA-2G maintained the definite stimulatory activity over 5 d with respect to both the relative rate of collagen synthesis (Fig. 5a) and the total amount of collagen secreted in the medium (Fig. 5b), but L-ascorbic acid at the same concentration lost the activity after 5-d cultivation. To examine the effect of AA-2G on the growth of fibroblasts, the cells were cultured for 24 d in the presence of AA-2G. As illustrated in Figure 6, the growth of the cells was obviously stimulated by continuous addition of 0.25 mmol/L AA-2G. The DNA content of the cells cultured for 24 d in the presence of AA-2G was 8.82 μg/dish, resulting in a fourfold greater value than that of the control cells.

## DISCUSSION

In this study, we observed that AA-2G, a new derivative of L-ascorbic acid, stimulated collagen synthesis in cultured skin fibroblasts and was more stimulatory than L-ascorbic acid. Thus, AA-2G is a very stable L-ascorbic acid source under the culture conditions employed and has a long-lasting effect due to its slow release of L-ascorbic acid. We also compared the effect of AA-2G on the stimulation of collagen synthesis with those of L-ascorbic acid-related compounds, including some chemically stable forms. Among them, AA-2P showed a similar stimulatory effect on collagen synthesis, whereas AA-2S and AA-6G had no or little effect. AA-2G as well as AA-2P

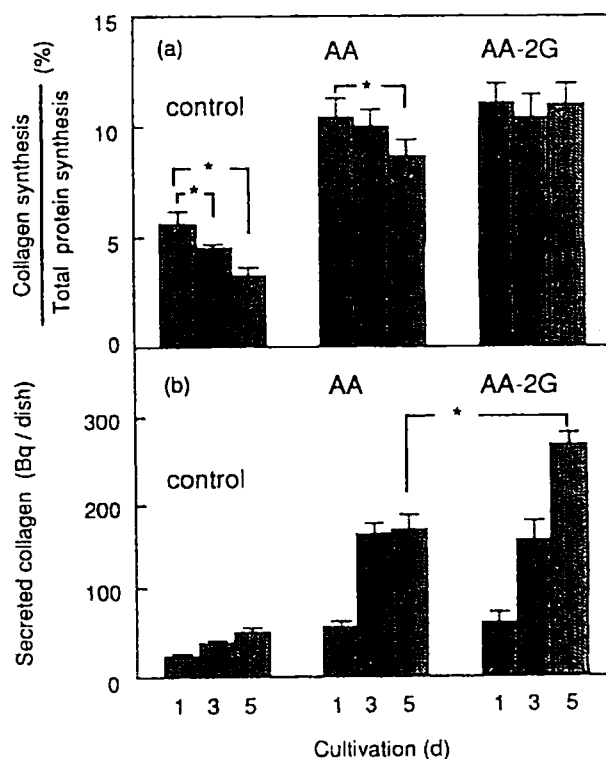


FIGURE 5 Longevity of stimulatory action of L-ascorbic acid (AA) and 2-*O*-α-D-glucopyranosyl-L-ascorbic acid (AA-2G) on collagen synthesis in human skin fibroblasts. The cells were incubated in 2.0 mL of fresh MEM-10 (minimum essential medium with 10% fetal bovine serum) containing [<sup>3</sup>H]proline (74 MBq/L) and each stimulator (0.25 mmol/L) for 1–5 d. The relative rate of collagen synthesis to total protein synthesis (a) and the total amount of collagen in the medium (b) were determined by the bacterial collagenase method. Points are the means ± SD of triplicate cultures. \*Significantly different than value in paired group ( $P < 0.05$ ).

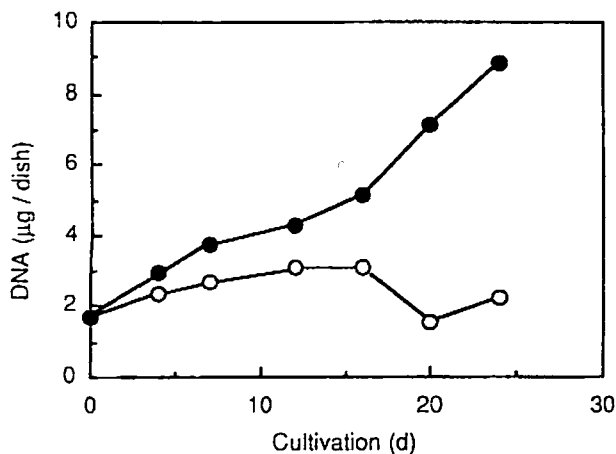


FIGURE 6 Effect of 2-O- $\alpha$ -D-glucopyranosyl-L-ascorbic acid (AA-2G) on proliferation of human skin fibroblasts. The cells ( $5 \times 10^4$ ) were cultured in the presence (●) or absence (○) of AA-2G (0.25 mmol/L) for 24 d at 37°C in 5% CO<sub>2</sub> air. The medium was changed twice a week. At the indicated incubation days, the medium was removed and the cell layer was processed for the determination of DNA content. Points are the means of duplicate cultures.

and AA-2S were nonreducing in their intact forms, although AA-6G had reducing power. Therefore, the difference in their abilities to enhance collagen synthesis *in vitro* may be explained by their availabilities in the cells. In fact, we observed relatively high  $\alpha$ -glucosidase and phosphatase activities to release L-ascorbic acid from AA-2G and AA-2P, respectively, and an extremely low level of hydrolyzing activity toward AA-2S in the cell lysate of cultured fibroblasts. In addition, we demonstrated previously (21) that AA-2G and AA-2P were effectively hydrolyzed by mammalian tissue homogenates to release L-ascorbic acid, whereas AA-2S and AA-6G were not. Those results support a correlation between the vitamin C activity of those stable derivatives and cellular hydrolytic activity toward them. This finding is consistent with those obtained in the *in vivo* determination of their antiscorbutic activities in guinea pigs and monkeys (7, 8, 11, 12).

It has been reported that AA-2G itself is unlikely to be absorbed through the intestine, considering its poor transport into the cell (21). In this study, we could not detect the intact form of AA-2G in the cultured fibroblasts by the present HPLC method. Therefore, to exhibit its vitamin C activity, AA-2G needs to be hydrolyzed before membrane transport. In fact, the addition of an  $\alpha$ -glucosidase inhibitor in the culture medium completely abolished the stimulatory effect of AA-2G. Because the existence of  $\alpha$ -glucosidase in bovine serum has been reported (29), we determined the stability of AA-2G in the culture medium containing fetal bovine serum. However, the participation of such a serum-derived enzyme can be

ruled out, because AA-2G was not hydrolyzed under these conditions. Taking these observations together with our previous findings (17), we consider membrane-bound neutral  $\alpha$ -glucosidase of the fibroblast to be responsible for the hydrolysis of AA-2G. Mammalian  $\alpha$ -glucosidases have been shown to hydrolyze the  $\alpha$ -1,4-,  $\alpha$ -1,3- and  $\alpha$ -1,2-glucosidic linkages more effectively than the  $\alpha$ -1,6-glucosidic linkages (29). Based on the structural similarity between disaccharides and glucosides of L-ascorbic acid, mammalian  $\alpha$ -glucosidase is considered to hydrolyze O-glucosidic bond in AA-2G more effectively than in AA-6G. Because AA-6G has a little improved stability in aqueous solution compared to L-ascorbic acid (16), its weak stimulatory effect on collagen synthesis may be primarily due to low hydrolysis by the cells.

It is an interesting finding in this study that only a part of AA-2G (<10% of AA-2G) in culture medium was utilized by fibroblasts and promoted collagen synthesis effectively. This result indicates that the amount of L-ascorbic acid inducing maximal collagen production is much lower than that added to the medium and that very little L-ascorbic acid is consumed for collagen synthesis in the cell. In fact, although 0.25 mmol/L of L-ascorbic acid completely disappeared from the culture medium by 60 h, the cells in 3-d cultivation maintained a high level of collagen synthesis. The ability of L-ascorbic acid to function at very low levels is supported by the observation by Blanck and Peterkofsky (30) that the proline hydroxylation reaction remained relatively unaffected when L-ascorbic acid levels declined drastically in guinea pigs fed diets without L-ascorbic acid. One possibility is that L-ascorbic acid is regenerated by the enzymatic system in the cell, so a low intracellular level of L-ascorbic acid may be adequate to promote full hydroxylation.

Hata and Senoo (9, 10) reported that AA-2P stimulated collagen accumulation, cell proliferation and formation of a three-dimensional tissue-like structure of skin fibroblasts. We also observed that cultured fibroblasts form a tissue-like structure after 1-wk culture in AA-2G-supplemented medium (data not shown). Thus, AA-2G supplementation may be useful for several prolonged cultivations with respect to the following: 1) AA-2G is a stable source of vitamin C activity; 2) AA-2G has no cell toxicity, making it possible to add enough AA-2G to the culture and promote higher collagen synthesis than AA; 3) AA-2G is easily hydrolyzed by cellular  $\alpha$ -glucosidase; and 4) AA-2G is a naturally occurring metabolite; it can be detected in blood and urine after a combined oral administration of L-ascorbic acid and maltose to rats and guinea pigs (31). Recently, cultured skin fibroblasts have been used for skin grafting. We expect that AA-2G is used in such a culture system as a durable L-ascorbic acid derivative. Furthermore, we propose its positive applications to several cell cultures for the production of useful substances and to

various multicomponent liquid products such as pharmaceuticals, foods and cosmetics.

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