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Ascorbic acid enhances the expression of type 1 and type 4 collagen and SVCT2 in cultured human skin fibroblasts

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ABSTRACT

Ascorbic acid (AA) is essential for collagen biosynthesis as a cofactor for prolyl and lysyl hydroxylase and as a stimulus for collagen gene expression. Many studies have evaluated the relationship between AA and collagen expression in short- and long-term effects on cells after a single administration of AA into the culture medium. However, no such study has monitored in detail the stability of AA in medium or the alterations of intracellular AA levels during a protracted interval. Therefore, we examined here intracellular AA levels and stability throughout its exposure to human skin fibroblasts *in vitro*. Moreover, we determined the effects on type 1 and type 4 collagen and sodium-dependent vitamin C transporter (SVCT) gene expression when medium containing 100 μM AA was replaced every 24 h for 5 days to avoid depletion of AA. Throughout this long-term culture, intracellular AA levels remained constant; the expression of type 1 and type 4 collagens and SVCT2 mRNA was enhanced, and type 1 procollagen synthesis increased. Thus, these results indicate that human skin fibroblasts exposed to AA over time had rising levels of type 1/type 4 collagens and SVCT2 mRNA expression and type 1 procollagen synthesis.

1. Introduction

Ascorbic acid (AA, Vitamin C) acts as an electron donor and reduces reactive oxygen species (ROS) such as superoxide radicals [1] and hydroxyl radicals [2]. AA also acts as a cofactor in nonheme iron and α-keglutarate-dependent dioxygenases [3]. Especially in collagen biosynthesis, AA is an essential cofactor of prolyl and lysyl hydroxylases for the hydroxylation of proline and lysine to form hydroxyproline and hydroxylysine [4]. Humans fail to synthesize AA *in vivo*, because they lack L-gulonolactone oxidase, which catalyzes the last step of the AA synthesis pathway [5]. Therefore, humans develop the symptoms of scurvy when ingesting an AA-free diet [5]. The AA deficiency is associated with a defect in connective tissues, particularly in wound healing, because these tissues cannot stabilize the triple helical structure of collagens [6]. Among the 29 types of collagen, type 1 collagen, in particular, is among the most abundant of these fibrous proteins in vertebrates, occupies most connective tissues, and is plentiful in bone, cornea, tendons, and dermis [7]. Type 1 collagen comprises a triple helix with two alpha 1 and one alpha 2 chains. On the other hand, type 4 collagen is one of the major components of the endothelial basement membrane in which it serves a structural function and interacts with other proteins of the basement membrane [8]. Hydroxyproline is essential for stabilizing the triple helical structure of collagens, and its absence prevents fibroblasts from excreting collagen to the outside mileau [9]. Similarly, hydroxylysine is essential in collagen cross-link formation, and its absence causes structural instability [10]. In addition to promoting these functions, AA has other beneficial effects in the skin. AA protects keratinocytes from ROS-mediated damage after ultraviolet B (UVB) irradiation *in vitro* [10]. Moreover, AA inhibits melanin production in melanocytes by reducing dopaquinone to dopa, which is a substrate of the melanin synthesis pathway. Recently, we reported that an AA deficiency leads to epidermal atrophy and extensive UVB-induced skin pigmentation. This result was achieved by using senescence marker

Abbreviations: AA, ascorbic acid; DHA, dehydroascorbic acid; ECD, electrochemical detection; EDTA, ethylenediaminetetraacetic acid; EIA, enzyme immunoassay; FBS, fetal bovine serum; GNL, gluconolactonase; HA, hyaluronic acid; HPLC, high performance liquid chromatography; KO, knockout; MPA, metaphosphoric acid; P1P, procollagen type 1 C-peptide; qPCR, quantitative real-time polymerase chain reaction; ROS, reactive oxygen species; SMP30, senescence marker protein-30; SVCT, sodium-dependent vitamin C transporter; UVB, ultraviolet B.

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protein-30 (SMP30)/gluconolactonase (GNL) knockout (KO) hairless mice, which cannot synthesize AA *in vivo*, because SMP30/GNL is an essential enzyme of the AA biosynthetic pathway [11].

AA migrates through cell membranes by using two specialized transporters, sodium-dependent vitamin C transporter (SVCT) 1 and 2. Especially in fibroblasts, which synthesize collagen, SVCT2 is important as the source of AA uptake from the extracellular fluid [12]. However, still unknown is whether AA affects SVCT2 mRNA expression levels in skin fibroblasts. We previously reported that AA depletion increased SVCT1 and SVCT2 mRNA expression levels in the liver and strengthened the AA uptake ability of hepatic cells [13].

Outcomes from several studies suggest that AA acts not only as a cofactor in the hydroxylation of proline and lysine but also as an enhancer of collagen gene expression in fibroblasts [9,14–16]. Of these regulatory factors of collagen gene expression, malondialdehyde, the end product of lipid peroxidation, is one of the most studied as a modulator of collagen. Malondialdehyde increases *Sp1* gene expression, and the Sp1 and Sp3 proteins bind the promoter region of the collagen α1(I) gene [17]. Moreover, collagen gene expression is also regulated both positively and negatively by a variety of exogenous factors including inflammatory response mediators [18]. Although many studies have evaluated the relationship between a single infusion of AA and collagen expression, no such experiments have been directed to the long-term effect on cells of AA, AA stability in culture medium, or intracellular AA levels. Therefore, we have now investigated the long-term effect of AA exposure on collagen expression by combining human skin fibroblasts with AA continuously replaced in culture medium every 24 h for 5 days.

2. Materials and methods

2.1. Cell culture

Human normal diploid skin fibroblasts, i.e., TIG-111 cells (JCRB0541), established from a skin biopsy of an apparently healthy human donor (female, 34 years old) were obtained from the Health Science Research Resources Bank (Osaka, Japan). Cells were cultured in minimal essential medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech Bio, Inc., Kerrville, TX, USA) at 37 °C under 5% CO_2 in air. Media were replaced every 3 or 4 days.

2.2. AA stability in the medium without cells

Various concentrations of AA (50, 100, and 500 μM) in media supplemented with 10% FBS were incubated at 37 °C under 5% CO_2 in air. Aliquots of medium were obtained at 1, 3, 6, 12, 24, 48, 72, 96, and 120 h for measurement of the AA concentration.

2.3. AA uptake study

Cells were plated into 60 mm diameter culture plates at 1×10^6 cells/ml and cultured overnight. To assess AA uptake for the first 24 h, cells were incubated with 50, 100, or 500 μM AA and without AA in the medium for 1, 3, 6, 12, 18, and 24 h. Subsequently, cells were incubated with and without 100 μM AA in the medium for 24, 48, 72, 96, and 120 h with the AA replaced every 24 h. At the appropriate times, cell were washed with phosphate buffered saline and collected with 5% metaphosphoric acid (MPA) to measure the AA content in cells. The protein content was measured by the method of Lowry et al. [19] using bovine serum albumin as a standard.

2.4. Measurement of AA and dehydroascorbic acid (DHA)

AA and DHA were measured by using high performance liquid chromatography (HPLC) and electrochemical detection (ECD) as described previously [20].

2.5. Extraction of total RNA and cDNA synthesis

Total RNA was extracted by using ISOGEN® (Wako Pure Chemical, Osaka, Japan) [21]. RNA concentrations were determined and confirmed as free from protein contamination by measuring absorbance at 260 and 280 nm. Then, cDNA was synthesized using SuperScript II Reverse transcriptase (Invitrogen) following the manufacturer's protocol. The cDNA was stored at −80 °C until use.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

Using the qPCR super mix-UDG-with ROX (Invitrogen) following the manufacturer's protocol, qPCR reactions were performed in triplicate. The primer for Collagen1A1 (Assay ID: Hs00164004_m1), Collagen4A1 (Assay ID: Hs00266237_m1), SVCT1 (Assay ID: Hs00195277_m1), SVCT2 (Assay ID: Hs00192765_m1) and GAPDH (Assay ID: Hs99999905_m1) came from an inventory of TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). As an endogenous control gene, TaqMan human GAPDH Control Reagent was used. The reactions were performed by using the 7300 Real Time PCR System (Applied Biosystems). The mRNA expression levels of type 1 and type 4 collagens and SVCT2 were evaluated relative to these levels of GAPDH, and the type 1 and type 4 collagens and SVCT2 mRNA expression levels in the fibroblasts without AA in the medium was considered as 100%.

2.7. Measurement of type 1 procollagen, hyaluronic acid (HA) and elastin

Type 1 procollagen was measured by using the Procollagen Type 1 C-peptide (PIP) enzyme immunoassay (EIA) Kit (Takara Bio, Kyoto, Japan) according to the manufacturer's instructions. HA was measured by using a Hyaluronan Enzyme-Linked Immunosorbent Assay kit (Echelon Bio, Salt Lake City, UT, USA) according to the manufacturer's instructions. Elastin was measured by using Fastin Elastin Assay kit (Biocolor Ltd., Carrickfergus, County Antrim, UK) according to the manufacturer's instructions.

2.8. Statistical analysis

The results are expressed as mean ± SEM. The probability of statistical differences between experimental groups was determined by Student's *t*-test using KaleidaGraph software (Synergy Software, Reading, PA, USA). Statistical differences were considered significant at $p < 0.05$.

3. Results

3.1. AA stability in the medium

We first examined the stability of AA in culture medium without cells during incubation at 37 °C. As shown in Fig. 1, AA levels in the medium decreased in a time-dependent manner. After 24 h of incubation, the initial AA levels of 50, 100, and 500 μM AA dropped to 2.0 ± 0.1, 8.5 ± 0.5, and 117.2 ± 3.0 μM, respectively. Moreover, AA was undetectable at 48 h in the medium first containing 50 or 100 μM AA, and after 72 h the starting amount of 500 μM AA vanished. On the other hand, DHA, the oxidized form

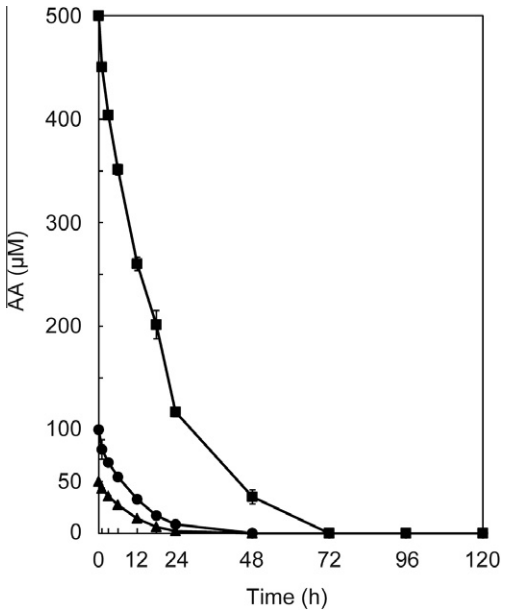

Fig. 1. AA stability in the medium without cells. AA (50 (▲), 100 (●), or 500 (■) μM) was added to medium supplemented with 10% FBS and incubated at 37 °C under 5% CO_2 in air. Aliquots of medium were obtained after 1, 3, 6, 12, 24, 48, 72, 96, and 120 h for measurement of AA concentrations. Values are expressed as means ± SEM of three plates.

of AA, remained detectable in the medium first incubated with 100 μM AA at approximate quantities of 3.1 to 5.4 μM until 24 h, i.e., the relative percentage of DHA (DHA/total AA) was higher in the medium during incubation (Supplemental Table S1).

3.2. AA uptake by fibroblasts

After human skin fibroblasts were incubated with 50, 100, or 500 μM AA for 1, 3, 6, 12, and 24 h, the cells uptake of AA was calculated. At all three initial doses, intracellular AA levels increased until 12 h and then gradually decreased until 24 h (Fig. 2A). At the 12 h peak time of uptake, initial AA levels of 50, 100, and 500 μM AA reached intracellular contents of 13.2 ± 1.2, 25.7 ± 1.1, 29.8 ± 1.2 μmol/g protein, respectively. Subsequent intracellular AA levels starting at 50, 100, and 500 μM AA remained at 6.3 ± 0.2, 18.6 ± 2.0, 24.6 ± 1.1 μmol/g protein, respectively, after 24 h. Simultaneously, AA levels in the medium were significantly reduced in a time-dependent manner until 24 h (Fig. 2B).

Next, for a 120 h assessment, cells were incubated with 100 μM AA for 24, 72, and 120 h, and the AA was replaced every 24 h until 120 h. Intracellular AA levels at 24, 72 and 120 h were 17.2 ± 1.5, 18.2 ± 0.9 and 18.0 ± 2.4 μmol/g protein, respectively, reflecting no significant differences of intracellular AA levels during these intervals (Fig. 2C). On the other hand, AA levels in the medium first containing 100 μM AA, which was replaced every 24 h, were 26.9 ± 1.1, 25.5 ± 0.8, 26.9 ± 0.3, 34.1 ± 0.3, and 22.7 ± 0.3 μM at 24, 48, 72, 96, and 120 h, respectively (Fig. 2D).

3.3. Morphology of human skin fibroblasts incubated with AA

A micrograph of the fibroblasts during incubation with and without 100 μM AA appears in Supplementary Figure S1. There were no cytotoxic or morphological differences between cultures with and without 100 μM AA throughout the entire 120 h period.

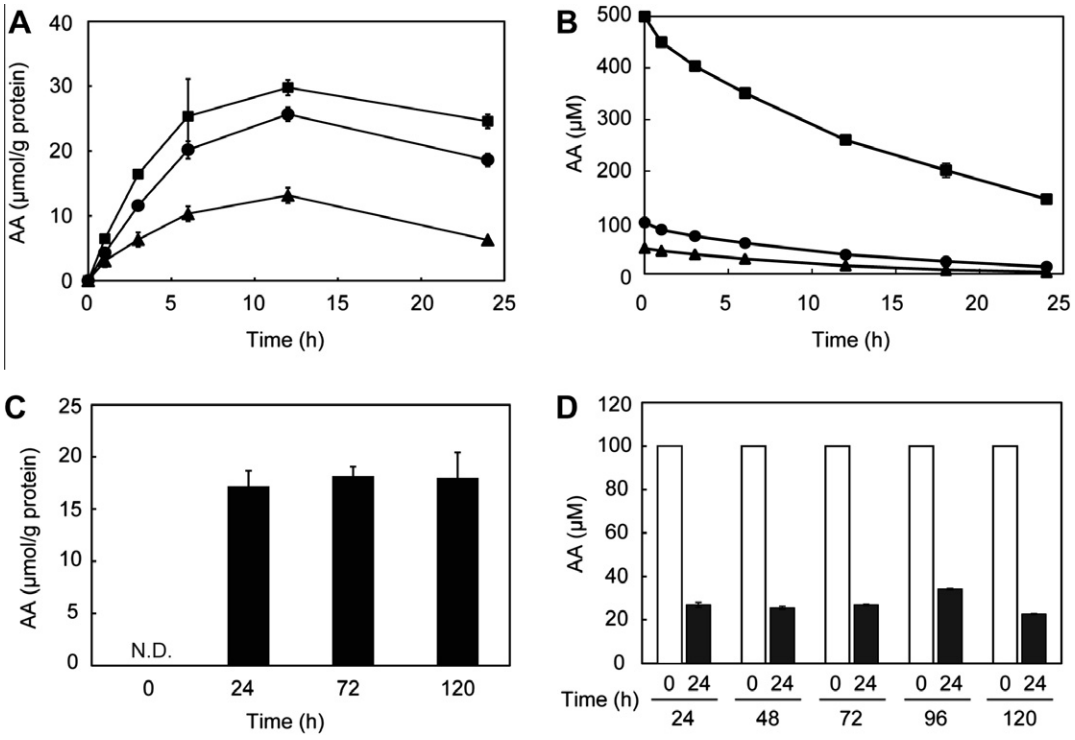

Fig. 2. AA uptake by human skin fibroblasts and extracellular AA levels during incubation. (A) Cells were incubated with 50 (▲), 100 (●), and 500 (■) μM AA for 1, 3, 6, 12, and 24 h and intracellular AA levels determined. (B) AA levels in medium treated with 50 (▲), 100 (●), or 500 (■) μM AA for 24 h. (C) Cells were incubated with 100 μM AA for 24, 72, and 120 h and intracellular AA levels determined. AA in the medium was replaced every 24 h. (D) Initial doses of 100 μM AA were replaced every 24 h during incubation lasting for 48, 72, 96, and 120 h. AA levels in the medium at 0 (□) and 24 h (■) at indicated time points before and after the addition of 100 μM AA throughout a 120 h period. Values are expressed as means ± SEM of three plates. N.D., not detected.

3.4. Effect of AA on type 1 and type 4 collagens and SVCT2 mRNA expression

To evaluate the effect of AA on type 1 and type 4 collagens and SVCT1 and SVCT2 mRNA expression levels, human skin fibroblasts were incubated with 100 μM AA for 24, 72, and 120 h and analyzed with the qPCR method. The amounts of type 1 collagen mRNA expressed at 24, 72, and 120 h were 1.2-, 1.6-, and 2.4-fold higher, respectively, than that of the same cells cultured without AA (Fig. 3A). Differences between cells either combined with 100 μM AA or not differed significantly at 72 and 120 h, but not at 24 h. The mRNA expression levels of type 4 collagen at 24, 72, and 120 h were 1.0-, 2.0-, and 3.1-fold higher than the cells without AA, respectively (Fig. 3B). The differences were also significant between cells cultured with and without 100 μM AA at 72 and 120 h, but not at 24 h. Moreover, the mRNA expression levels of SVCT2 at 24, 72, and 120 h were 1.6-, 1.5-, and 1.3-fold higher than the cells without AA, respectively (Fig. 3C). There was a significant difference between cultures with and without 100 μM AA at 24 and 72 h, but not at 120 h. However, regardless whether AA was present in or absent from the medium, no SVCT1 mRNA at all was detectable in these human skin fibroblasts.

3.5. Effect of AA on type 1 procollagen synthesis, HA synthesis and elastin synthesis

To confirm the effect of AA on type 1 procollagen synthesis, we incubated fibroblasts with or without 100 μM AA and measured the amounts of procollagen type 1 C-peptide secreted into the medium from 0 to 24 h, from 48 to 72 h, and from 96 to 120 h. The amounts of type 1 procollagen released into the medium during the first 24 h and from 48 to 72 h, as well as 96 to 120 h were a significant 2.8-, 3.8-, and 8.0-fold higher than that without AA, respectively (Fig. 4A).

We then measured the HA levels in medium containing AA. No significant difference was evident between HA levels in culture medium incubated with or without 100 μM AA for 24 h (Fig. 4B).

Finally, the possible effect of AA on elastin synthesis was assessed. Treatment with 100 μM AA for 24, 72, and 120 h produced no significant difference when compared to AA-negative cultures (Fig. 4C).

4. Discussion

In the present study, we confirmed that AA remained stable during incubation in the presence of human skin fibroblasts and was effectively taken up by these cells in long-term culture. Intracellular AA levels remained constant throughout this long-term culture for 120 h. Further, during this prolonged exposure of up to 120 h, the expression of type 1 and type 4 collagens and SVCT2 escalated. In accord, type 1 procollagen synthesis increased. However, neither HA nor elastin synthesis was affected by the presence of AA.

We first monitored the stability of AA in culture medium, because AA has a potent reducing ability and oxidizes to DHA. DHA

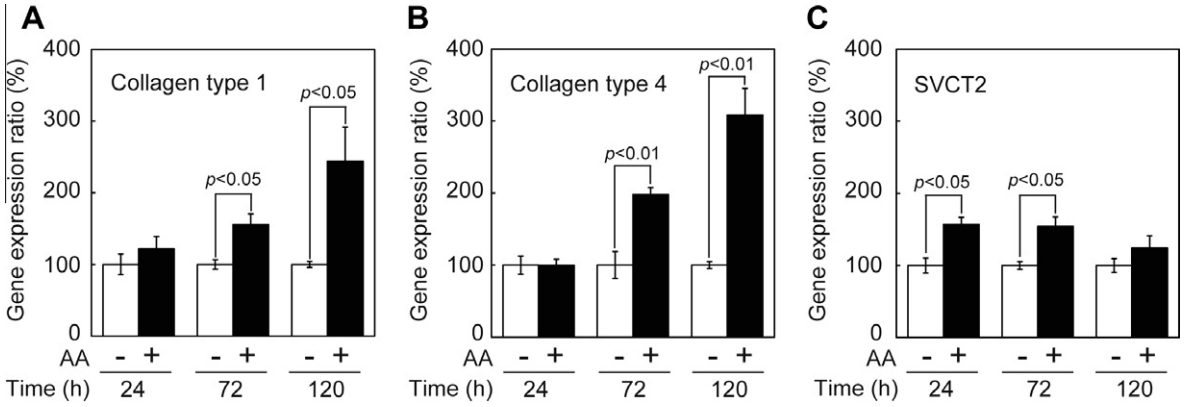

Fig. 3. Effect of AA on type 1 and 4 collagen and SVCT2 mRNA expression in human skin fibroblasts treated with 100 μM AA (■) and without AA (□). Type 1 collagen (A), Type 4 collagen (B), and SVCT2 (C) mRNA expression levels were measured by qPCR. The mRNA expression of type 1 and type 4 collagens and SVCT2 was evaluated relative to these levels of GAPDH, and type 1 and type 4 collagens and SVCT2 mRNA expression in fibroblasts without AA in the medium was considered as 100%. Values are expressed as means ± SEM of three plates.

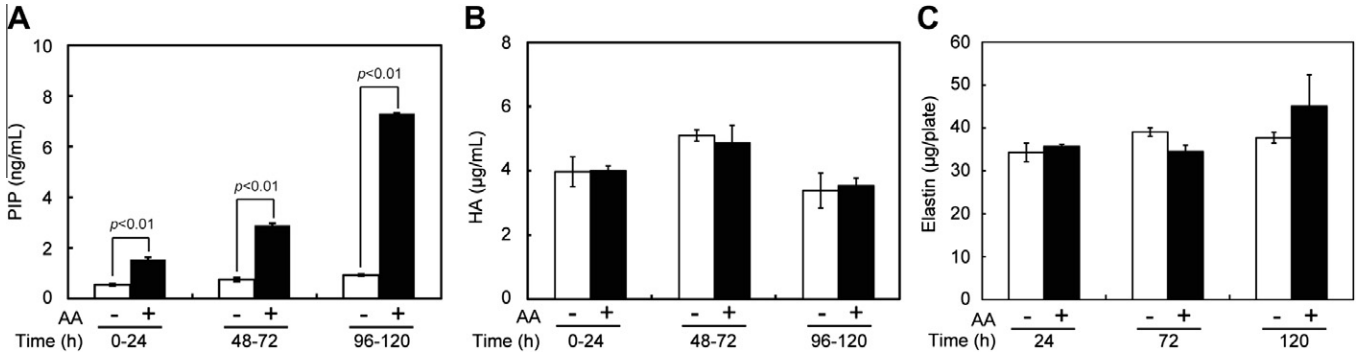

Fig. 4. Effect of AA on type 1 procollagen, HA and elastin synthesis. (A) Values for type 1 procollagen incubated with 100 μM AA (■) and without AA (□) were determined by using a P1P EIA. P1P levels in the culture medium were assessed from 0 to 24 h, from 48 to 72 h, and from 96 to 120 h. (B) HA incubated with 100 μM AA (■) and without AA (□) was evaluated by HA EIA. HA levels in the culture medium were assessed from 0 to 24 h, from 48 to 72 h, and from 96 to 120 h. (C) Levels of α-elastin incubated with 100 μM AA (■) and without AA (□) were determined by using Fastin Elastin Assays. Intracellular elastin levels were appraised at 24, 72, and 120 h. Values are expressed as means ± SEM of three plates.

is irreversibly hydrolyzed to 2,3-diketoguronic acid at neutral pH [22,23]. In this study, AA levels decreased at 24 h after incubation in medium without cells, and AA first instilled at 50, 100, and 500 μM fell to levels of 4, 9, and 29%, respectively, of their starting values. Moreover, when 50 or 100 μM AA were added to the medium, no AA at all was detected 48 h later, and in medium initially containing 500 μM, AA was no longer detectable at 72 h. In contrast, the relative percentage of DHA increased in a time-dependent manner, although the net DHA level was not changed until 24 h elapsed. These results suggest that AA might be degraded not only by the oxidation–reduction reaction but also by another degradative pathway.

To investigate a continuous effect of AA on cells, culture medium containing 100 μM AA was replaced every 24 h during a 5-day experimental period to avoid the AA's depletion. This is the first study documenting a continuous effect of AA on human skin fibroblasts. Intracellular AA levels reached a peak at 12 h when the culture medium initially contained 50, 100, or 500 μM AA. Moreover, the peak intracellular AA level of fibroblasts combined with 100 μM AA was 2.0-fold higher than that with 50 μM AA. However, the peak AA concentration of fibroblasts mixed with 500 μM AA was only 1.2-fold higher than that treated with 100 μM AA. These results suggest that intracellular AA levels reached a plateau at or near a 30 μmol/g protein in human skin fibroblasts. After the intracellular AA content peaked at 12 h, that amount dropped by 24 h of culture, even though the starting concentration was the highest dose of 500 μM AA. Although cell culture conditions and maximum AA levels in these cells differed in a report by Duarte and Jones [24], the kinetics of AA transportation resembled ours. In the present study, fibroblasts in medium reconstituted with 100 μM AA every 24 h maintained almost same intracellular AA levels for 5 days.

It is known that AA uptake from the extracellular environment occurs via SVCT1 and SVCT2, both of which are 12-transmembrane proteins [25]. SVCT1 and SVCT2 have functional roles in sodium-dependent and secondary active transport of AA from the outside to interiors of cells [26]. In our hands, qPCR analysis failed to detect any SVCT1 mRNA at all in human skin fibroblasts. Yet, after long-term exposure amounting to 24- and 72-h incubation with AA, the mRNA expression levels of SVCT2 in cells were significantly higher than that of cells without AA. On the other hand, we previously reported that AA depletion enhanced SVCT1 and SVCT2 mRNA expression levels in the livers of SMP30/GNL KO mice [13]. Thus, SVCT might behave differentially among various organs with respect to cells incorporation of AA.

The expression of type 1 and type 4 collagen mRNAs was not significantly different for cells combined with 100 μM AA or without AA at 24 h. However, these mRNA expression levels of cells incubated with 100 μM AA were significantly higher than that of cells without AA at 72 and 120 h. Thus, long-term AA exposure heightened the expression of type 1 and type 4 collagen mRNA in human skin fibroblasts. Moreover, we confirmed that AA enhanced type 1 procollagen synthesis. In particular, type 1 procollagen synthesis, as measured in medium containing 100 μM AA for 24 h, was a significant 8-fold higher from 96 to 120 h of culture than that without AA. In the same situation, type 1 procollagen synthesis increased in the first 24 h of incubation despite no difference in the expression of type 1 collagen mRNA at 24 h, as determined by qPCR. The cause of this time difference might be that immature type 1 procollagen present in the cells before culturing was secreted during AA exposure but before induction of its ability to express the mRNA. In addition to this result, we previously reported that a complete lack of AA decreased type 1 collagen mRNA expression in lungs and caused emphysema in SMP30/GNL KO mice [27].

In conclusion, after long-term exposure to AA, human skin fibroblasts maintained a constant intracellular AA level. The expression of type 1 and type 4 collagens and SVCT2 mRNAs was enhanced as was type 1 procollagen synthesis in human skin fibroblasts. This first trial and first report of AA's continuous effect on human skin fibroblasts may foreshadow new and direct evidence of AA's therapeutic usefulness.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.11.110.

References

[1] M. Nishikimi, Oxidation of ascorbic acid with superoxide anion generated by the xanthine-xanthine oxidase system, Biochem. Biophys. Res. Commun. 63 (1975) 463–468.
[2] B.H. Bielski, H.W. Richter, P.C. Chan, Some properties of the ascorbate free radical, Ann. N.Y. Acad. Sci. 258 (1975) 231–237.
[3] R.P. Hausinger, Fe(II)/α-ketoglutarate-dependent hydroxylases and related enzymes, Crit. Rev. Biochem. Mol. Biol. 39 (2004) 21–68.
[4] B. Peterkofsky, S. Udenfriend, Enzymatic hydroxylation of proline in microsomal polypeptide leading to formation of collagen, Proc. Natl. Acad. Sci. USA 53 (1965) 335–342.
[5] M. Nishikimi, T. Koshizaka, T. Ozawa, K. Yagi, Occurrence in humans and guinea pigs of the gene related to their missing enzyme L-gulono-γ-lactone oxidase, Arch. Biochem. Biophys. 267 (1988) 842–846.
[6] B. Peterkofsky, Ascorbate requirement for hydroxylation and secretion of procollagen: relationship to inhibition of collagen synthesis in scurvy, Am. J. Clin. Nutr. 54 (1991) 1135S–1140S.
[7] G.A. Di Lullo, S.M. Sweeney, J. Korkko, L. Ala-Kokko, J.D. San Antonio, Mapping the ligand-binding sites and disease-associated mutations on the most abundant protein in the human, type I collagen, J. Biol. Chem. 277 (2002) 4223–4231.
[8] B.G. Hudson, S.T. Reeders, K. Tryggvason, Type IV collagen: structure, gene organization, and role in human diseases. Molecular basis of Goodpasture and Alport syndromes and diffuse leiomyomatosis, J. Biol. Chem. 268 (1993) 26033–26036.
[9] S.R. Pinnell, Regulation of collagen biosynthesis by ascorbic acid: a review, Yale J. Biol. Med. 58 (1985) 553–559.
[10] M.S. Stewart, G.S. Cameron, B.C. Pence, Antioxidant nutrients protect against UVB-induced oxidative damage to DNA of mouse keratinocytes in culture, J. Invest. Dermatol. 106 (1996) 1086–1089.
[11] Y. Sato, K.Y. Arai, T. Nishiyama, Y. Nomura, Y. Kishimoto, S. Aizawa, N. Maruyama, A. Ishigami, Ascorbic acid deficiency leads to epidermal atrophy and UVB-induced skin pigmentation in SMP30/GNL knockout hairless mice, J. Invest. Dermatol. 132 (2012) 2112–2115.
[12] H. Steiling, K. Longet, A. Moodycliffe, R. Mansourian, E. Bertschy, H. Smola, C. Mauch, G. Williamson, Sodium-dependent vitamin C transporter isoforms in skin: distribution, kinetics, and effect of UVB-induced oxidative stress, Free Radical Biol. Med. 43 (2007) 752–762.
[13] A. Amano, T. Aigaki, N. Maruyama, A. Ishigami, Ascorbic acid depletion enhances expression of the sodium-dependent vitamin C transporters, SVCT1 and SVCT2, and uptake of ascorbic acid in livers of SMP30/GNL knockout mice, Arch. Biochem. Biophys. 496 (2010) 38–44.
[14] K.P. Houglum, D.A. Brenner, M. Chojkier, Ascorbic acid stimulation of collagen biosynthesis independent of hydroxylation, Am. J. Clin. Nutr. 54 (1991) 1141S–1143S.
[15] T.L. Duarte, M.S. Cooke, G.D. Jones, Gene expression profiling reveals new protective roles for vitamin C in human skin cells, Free Radical Biol. Med. 46 (2009) 78–87.
[16] S. Kurata, H. Senoo, R. Hata, Transcriptional activation of type I collagen genes by ascorbic acid 2-phosphate in human skin fibroblasts and its failure in cells from a patient with α2(I)-chain-defective Ehlers–Danlos syndrome, Exp. Cell Res. 206 (1993) 63–71.
[17] I. Garcia-Ruiz, P. de la Torre, T. Diaz, E. Esteban, I. Fernandez, T. Munoz-Yague, J.A. Solis-Herruzo, Sp1 and Sp3 transcription factors mediate malondialdehyde-induced collagen α1(I) gene expression in cultured hepatic stellate cells, J. Biol. Chem. 277 (2002) 30551–30558.
[18] S.L. Adams, Collagen gene expression, Am. J. Respir. Cell Mol. Biol. 1 (1989) 161–168.
[19] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265–275.

[20] Y. Sato, T. Uchiki, M. Iwama, Y. Kishimoto, R. Takahashi, A. Ishigami, Determination of dehydroascorbic acid in mouse tissues and plasma by using tris(2-carboxyethyl)phosphine hydrochloride as reductant in metaphosphoric acid/ethylenediaminetetraacetic acid solution, Biol. Pharm. Bull. 33 (2010) 364–369.
[21] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem. 162 (1987) 156–159.
[22] A.M. Bode, L. Cunningham, R.C. Rose, Spontaneous decay of oxidized ascorbic acid (dehydro-L-ascorbic acid) evaluated by high-pressure liquid chromatography, Clin. Chem. 36 (1990) 1807–1809.
[23] H. Tanaka, E. Kimoto, Preparation and characterization of a 345 nm absorbing reductant derived from dehydro-L-ascorbic acid, Bull. Chem. Soc. Jpn. 63 (1990) 2569–2572.
[24] T.L. Duarte, G.D. Jones, Vitamin C modulation of H_2O_2-induced damage and iron homeostasis in human cells, Free Radic. Biol. Med. 43 (2007) 1165–1175.
[25] H. Tsukaguchi, T. Tokui, B. Mackenzie, U.V. Berger, X.Z. Chen, Y. Wang, R.F. Brubaker, M.A. Hediger, A family of mammalian Na^+-dependent L-ascorbic acid transporters, Nature 399 (1999) 70–75.
[26] W.J. Liang, D. Johnson, S.M. Jarvis, Vitamin C transport systems of mammalian cells, Mol. Membr. Biol. 18 (2001) 87–95.
[27] K. Koike, Y. Kondo, M. Sekiya, Y. Sato, K. Tobino, S.I. Iwakami, S. Goto, K. Takahashi, N. Maruyama, K. Seyama, A. Ishigami, Complete lack of vitamin C intake generates pulmonary emphysema in senescence marker protein-30 knockout mice, Am. J. Physiol. Lung Cell. Mol. Physiol. 298 (2010) L784–L792.