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Effects of L-ascorbic acid 2-phosphate magnesium salt on the properties of human gingival fibroblasts

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Background and Objective: L-Ascorbic acid 2-phosphate magnesium salt (APM) is an L-ascorbic acid (AsA) derivative developed to improve AsA stability and display effective biochemical characteristics. This study aimed to investigate the effects of APM on the functions and properties of human gingival fibroblasts with respect to the prevention of periodontal disease in comparison with those of AsA.

Material and Methods: Human gingival fibroblasts were incubated in the presence or absence of APM or L-ascorbic acid sodium salt (AsANa). Intracellular AsA was analysed by HPLC. Collagen synthesis was measured by ELISA and real-time RT-PCR. Intracellular reactive oxygen species (ROS) induced by hydrogen peroxide (H_2O_2) were quantified using a fluorescence reagent, and cell damage was estimated with calcein acetoxymethyl ester. Furthermore, intracellular ROS induced by tumor necrosis factor-α (TNF-α) were quantified, and expression of TNF-α-induced interleukin-8 expression, which increases due to inflammatory reactions, was measured by ELISA and real-time RT-PCR.

Results: APM remarkably and continuously enhanced intracellular AsA and promoted type 1 collagen synthesis and mRNA expression. Furthermore, APM decreased cell damage through the suppression of H_2O_2-induced intracellular ROS and inhibited interleukin-8 production through the suppression of TNF-α-induced intracellular ROS. These effects of APM were superior to those of AsANa.

Conclusion: These results suggest that APM is more effective than AsANa in terms of intake, collagen synthesis, decreasing cell damage and inhibiting interleukin-8 expression in human gingival fibroblasts. This suggests that local application of APM can help to prevent periodontal disease.

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L-Ascorbic acid (AsA), better known as vitamin C, has various biochemical functions, such as collagen synthesis in skin fibroblasts (1,2), phagocytosis of polymorphonuclear leukocytes (3), differentiation of several mesenchymal cell types (4) and antioxidant scavenging of reactive oxygen species (ROS; 5). However, AsA is highly unstable in aerobic conditions (6,7), neutral pH (8) and in solution (9).

L-Ascorbic acid 2-phosphate magnesium salt (APM) is an AsA derivative developed to improve AsA

stability. APM is highly resistant to degradation into AsA, even at neutral pH (10), but displays numerous biochemical characteristics, such as easy degradation into AsA in the presence of phosphatase from living tissues. Enhancement of collagen production in keratocytes (11) and skin fibroblasts (12) by APM has been reported. Furthermore, Kobayashi *et al.* (13) have demonstrated that APM can protect against lipid peroxidation and inflammation in cutaneous tissue induced by ultraviolet B exposure. Collagen synthesis by APM and its antioxidant properties are especially attractive to the skincare field.

Interestingly, these two functions are also required in gingival tissues for preventing periodontal disease. Human gingival fibroblasts are the major constituents of gingival tissues and maintain their homeostasis by regulating collagen metabolism (14). However, collagen in gingiva is degraded by MMP produced by periodontal pathogens or various host cells in inflamed periodontal tissues (15). Moreover, it has been reported that ROS, which play a major role in the etiology of periodontal disease, are induced by respiratory bursts of neutrophils (5,16) or by the proinflammatory signaling pathway (17–19). Many studies have indicated that ROS induce direct tissue destruction, such as collagen degradation and cell damage (20,21), or stimulate proinflammatory processes (22–24).

Therefore, the promotion of collagen synthesis and suppression of ROS by APM could be expected to contribute to the integrity of gingival tissues and prevention of periodontal disease. The aim of this study was to investigate the effects of APM on intake, collagen synthesis, and antioxidant and anti-inflammatory properties of human gingival fibroblasts in comparison with those of AsA.

Material and methods

Reagents

L-Ascorbic acid sodium salt (AsANa) was obtained from Wako Pure Chemical Industries (Osaka, Japan). APM was obtained from Showa Denko Co., Ltd (Tokyo, Japan). Hydrogen peroxide (H_2O_2) was obtained from Merck KgaA (Darmstadt, Germany). Calcein acetoxymethyl ester (calcein-AM) was obtained from Invitrogen Corporation (Carlsbad, CA, USA). Tumor necrosis factor-α (TNF-α) was obtained from R&D Systems Inc. (Minneapolis, MN, USA).

Cell culture

The protocol was reviewed and approved by the Institutional Review Board of the Osaka University Graduate School of Dentistry, and informed consent was obtained from all subjects participating in the study. Two cell lines of human gingival fibroblasts (HGF 1 and HGF 2) were obtained from biopsies of healthy gingiva from healthy volunteers as previously described (25). Cells of another human gingival fibroblast cell line, Gin-1, were obtained from DS Pharma Biomedical Co., Ltd (Osaka, Japan). Human gingival fibroblasts were grown in Dulbecco's modified Eagle's medium (DMEM; GIBCO, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS; SAFC Biosciences, Lenexa, KS, USA) and routinely passaged by trypsinization when nearly confluent. The HGF 1 cells were the main cell line evaluated in this study, and other cell lines exhibited similar tendencies to this cell line. The following experiments were performed after seeding the cells onto a culture plate (6.7×10^3 cells/cm^2) and pre-incubating them for 24 h in DMEM containing 10% FCS.

Content of AsA and APM in human gingival fibroblasts

Human gingival fibroblasts were incubated in 75 cm^2 flasks with 50 μM AsANa or APM in DMEM containing 1% FCS for 12–48 h. To determine whether APM was degraded into AsA by phosphatase present in FCS, APM was diluted in a medium containing heat-inactivated FCS at 56°C for 30 min in the APM (inactivated) group. Human gingival fibroblasts were washed three times with phosphate-buffered saline after incubation and collected in 0.1 mL of cold 5% metaphosphoric acid by scraping. The cell suspensions were sonicated and centrifuged at 10,000 *g* for 15 min at 4°C. The cell extract supernatants were maintained at −80°C until ready for HPLC analysis. In brief, the separation of AsA from the supernatants was achieved by isocratic elution from an Inertsil® ODS-3 column (4.6 mm × 250 mm, particle size 5 μm; GL Science, Tokyo, Japan), which was kept at 40°C, with 25 mM KH_2PO_4–H_3PO_4 buffer (pH 2.0) containing 20 μM EDTA and 25 mM tetrabutylammonium hydrogen sulfate at a flow rate of 0.8 mL/min. AsA was detected by an electrochemical detector (Eicom, Kyoto, Japan) adjusted to 550 mV, and APM was detected by a UV-Vis detector (Shimadzu, Kyoto, Japan) at 240 nm.

Cell proliferation

Cell proliferation was assessed by the trypan blue dye exclusion assay. Human gingival fibroblasts were incubated in a six-well plate with 50 μM AsANa or APM in DMEM containing 1% FCS for 12–72 h. The number of living cells was counted under microscopic observation after harvesting by trypsinization and staining with trypan blue (Invitrogen Corporation).

Collagen synthesis

Human gingival fibroblasts were incubated in a six-well plate with 50 μM AsANa or APM in DMEM containing 1% FCS for 3–72 h. Subsequently, the amount of type 1 collagen produced by human gingival fibroblasts in the culture medium supernatant was determined in each well using the Human Type 1 Collagen ELISA Kit (ACBio, Kanagawa, Japan) according to the manufacturer's instructions.

Extraction of RNA and real-time RT-PCR

Total RNA from human gingival fibroblasts was isolated using the RNeasy Mini Kit (QIAGEN K.K., Tokyo, Japan), and first-strand cDNA was synthesized using random primer

hexamers and M-MLV reverse transcriptase (Invitrogen Corporation). Real-time RT-PCR was performed using the Power PCR SYBR Master Mix (Applied Biosystems, Carlsbad, CA, USA) and gene-specific primers (Sigma-Aldrich Japan, K.K., Genosys Division, Ishikari, Japan) in a 7300 fast real-time RT-PCR system (Applied Biosystems) according to the manufacturer's instructions. Amplification conditions consisted of an initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s, and annealing and elongation at 60°C for 60 s. The primer sequences were as follows: glyceraldehyde-3-phosphate dehydrogenase (GAPDH), 5′-GCACCGTCAAGGCTGAGAAC-3′ (forward), 5′-ATGGTGGTGAAGACGCCAGT-3′ (reverse); type 1 collagen, 5′-CTGCTGGACGTCCTGGTGAA-3′ (forward), 5′-ACGCTGTCCAGCAATACCTTGAG-3′ (reverse); and interleukin-8 (IL-8), 5′-ACACTGCGCCAACACAGAAATTA-3′ (forward), 5′-TTTGCTTGAAGTTTCACTGGCATC-3′ (reverse). Relative expression was obtained after normalization with gene expression of GAPDH.

Intracellular ROS

Generation of intracellular ROS induced by H_2O_2 or TNF-α stimulation was evaluated using a ROS detection reagent, 5-(and-6)-chloromethyl-2′, 7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA; Molecular Probes, Carlsbad, CA, USA). In brief, this reagent is hydrolysed by intracellular esterases and is converted to the highly fluorescent derivative 5-(and-6)-chloromethyl-2′,7′-dichlorofluorescein through its reaction with intracellular ROS. The quantification of fluorescence intensity enabled us to measure intracellular ROS. Human gingival fibroblasts were incubated in a 24-well plate with 50 μM AsANa or APM in DMEM containing 1% FCS for 12–72 h. Incubated cells were exposed to 10 μM CM-H_2DCFDA in serum-free DMEM for 30 min and washed with phosphate-buffered saline to eliminate the extracellular reagent. Subsequently, the cells were stimulated by 1 mM H_2O_2 in serum-free DMEM for 0.5 h. The intracellular fluorescence intensity was detected at 485 nm excitation and 530 nm emission using a fluorescence plate reader. Intracellular ROS levels were calculated by integrating the area under the fluorescence intensity–time curve and converted to the relative value compared with that in the samples without H_2O_2 stimulation. Transmitted and fluorescent light images of the cells were observed, and the fluorescence intensity of each fluorescence image was quantified by an IN Cell Analyzer 1000 (GE Healthcare Japan, Tokyo, Japan) and converted to a relative value against the control. Likewise, evaluation of intracellular ROS after 8 h of stimulation by TNF-α was performed using the CM-H_2CDFDA reagent.

Cell viability assay

Cell viability was determined on the basis of the metabolism of calcein-AM, which produces a green fluorescence on reaction with intracellular esterase. Human gingival fibroblasts incubated for 24 h in the presence of AsANa or APM were exposed to 1 μM calcein-AM in Dulbecco's phosphate-buffered saline for 30 min after stimulation with 1 mM H_2O_2 for 0.5–2 h. Fluorescence intensity was detected using a fluorescence spectrometer, and cell viability (as a percentage) was calculated as follows: (fluorescence intensity of each group after H_2O_2 stimulation)/(fluorescence intensity without H_2O_2 stimulation) × 100.

Determination of IL-8 production

Human gingival fibroblasts were incubated in a 24-well plate with 50 μM AsANa or APM in DMEM containing 1% FCS for 24 h. The cells were stimulated with 1 nM TNF-α in DMEM containing 1% FCS. After stimulation for 8 h, total RNA was isolated from the cells, and IL-8 mRNA expression was measured. Finally, the culture medium supernatants were assayed for IL-8 using the Human IL-8 ELISA Kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Statistical analysis

ANOVA was used to compare groups, and the homogeneity of variance was confirmed by Bartlett's test. Tukey's multiple comparison test was used to determine differences among the three groups (control, AsANa and APM) with respect to cell proliferation, collagen synthesis, intracellular ROS, cell viability and IL-8 production. Unpaired student's *t*-test or the Aspin–Welch test was used to evaluate the changes in intracellular ROS and the amount of IL-8 produced in response to H_2O_2 or TNF-α stimulation.

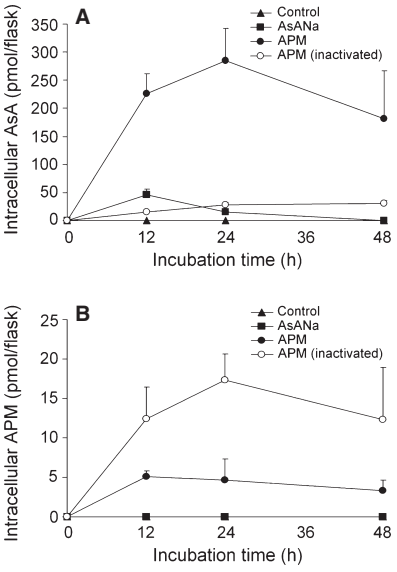

Fig. 1. The intracellular accumulation of L-ascorbic acid (AsA) and L-ascorbic acid 2-phosphate magnesium salt (APM) in human gingival fibroblasts. Human gingival fibroblasts were incubated in the presence or absence (control) of 50 μM of the sodium salt of AsA (AsANa) or APM in Dulbecco's modified Eagle's medium (DMEM) containing 1% fetal calf serum (FCS). Dulbecco's modified Eagle's medium containing 1% heat-inactivated FCS was used in the APM (inactivated) group. After incubation for 12–48 h, cell suspensions collected in 5% metaphosphoric acid were sonicated and centrifuged. The amount of intracellular AsA and APM in the supernatants was determined by HPLC. (A) AsA was detected by an electrochemical detector. (B) APM was detected by an UV-Vis detector. The results represent the means ± SD of three independent experiments.

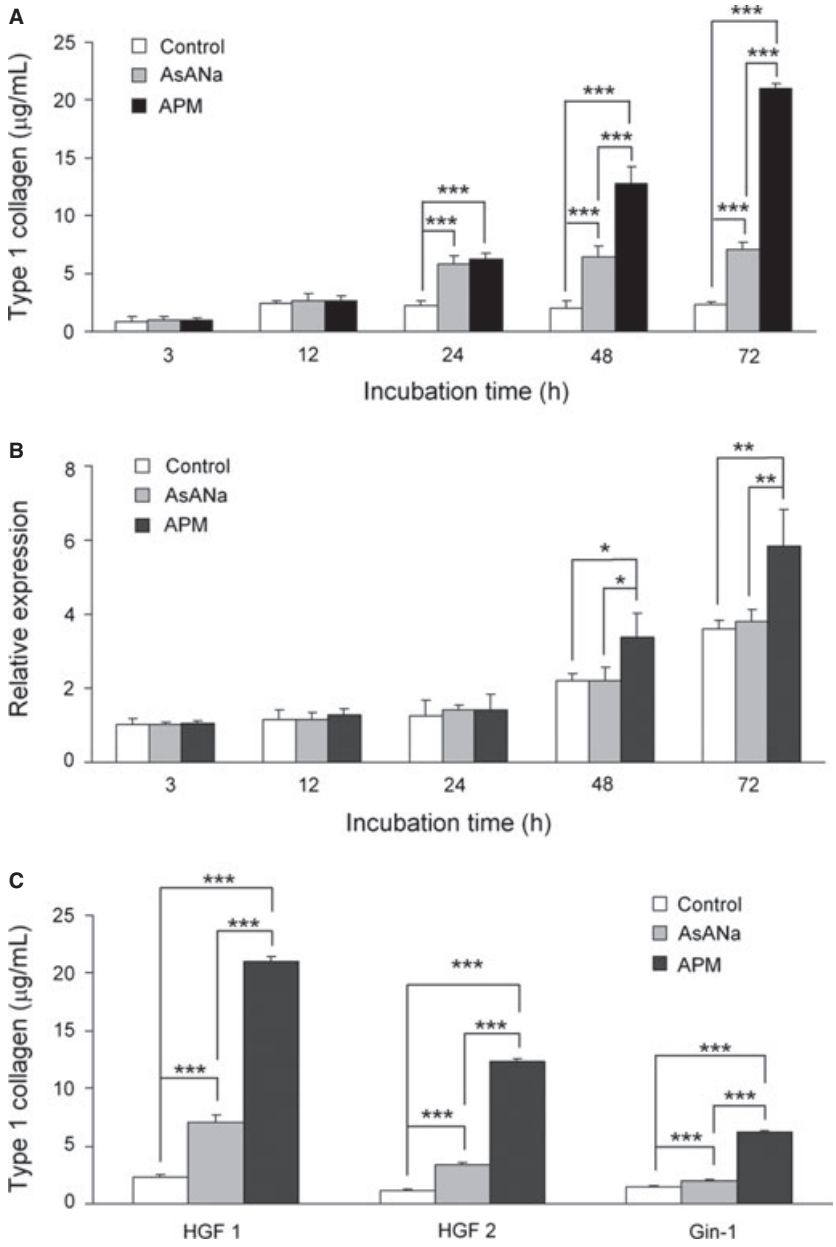

Fig. 2. Effects of AsANa and APM on collagen synthesis in human gingival fibroblasts. Human gingival fibroblasts were incubated in the presence or absence (control) of 50 μM AsANa or APM in DMEM containing 1% FCS for 12–72 h. (A) Cell culture supernatants of HGF 1 cells were measured using an ELISA kit. The results are shown as the means + SD ($n = 5$) of three independent experiments. (B) Type 1 collagen mRNA expression in HGF 1 cells was measured by real-time RT-PCR as described in the Material and methods section. The results are shown as the means + SD ($n = 4$) of three independent experiments. (C) After incubation for 72 h, the cell culture supernatants of HGF 1, HGF 2 and Gin-1 cells were measured using an ELISA kit. The results are shown as the means + SD ($n = 5$) of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Tukey's test.

Results

The amount of intracellular AsA was remarkably enhanced by APM

To clarify differences in intake of AsA by human gingival fibroblasts between AsANa and APM groups, we quantified the amount of AsA and APM in the supernatant derived from crushed cells. The APM group showed a remarkably enhanced level of intracellular AsA (> 200 pmol per flask) at 12 h, and this level was maintained up to 48 h; although levels were within 30 pmol in the APM (inactivated) group in which phosphatase in FCS was inactivated (Fig. 1A). Conversely, intracellular AsA in the AsANa group disappeared at 48 h, although a peak value of 50 pmol was observed at 12 h. There was no intracellular AsA detected in the control group. The

amounts of intracellular APM in the APM and APM (inactivated) groups were approximately 5 and 15 pmol, respectively, up to 48 h (Fig. 1B).

APM promoted type 1 collagen synthesis and mRNA expression in a time-dependent manner

To investigate the effects of AsANa and APM on collagen synthesis in human gingival fibroblasts, we measured the amount of type 1 collagen synthesized by HGF 1 cells in the supernatant of the culture medium. No difference in collagen synthesis was observed among the three groups until 12 h of incubation, but the AsANa and APM groups showed a significant increase compared with the control group after 24 h (Fig. 2A). Collagen synthesis continued in the APM group and accelerated after 48 h, with a twofold higher level than that in the AsANa group at 48 h ($p < 0.001$) and a threefold higher level at 72 h ($p < 0.001$). To elucidate the cause of this acceleration, we evaluated type 1 collagen mRNA expression. The APM group showed significantly higher type 1 collagen mRNA expression at 48 ($p < 0.05$) and 72 h ($p < 0.01$) than that observed in the control and AsANa groups, whereas type 1 collagen mRNA expression in the AsANa group did not differ from that in the control group (Fig. 2B). This acceleration of collagen synthesis was also observed in HGF 2 and Gin-1 cells (Fig. 2C). However, no significant difference in the proliferation of HGF 1 cells was observed among the three groups at any incubation time point.

APM decreased cell damage by suppressing intracellular ROS produced by H_2O_2 stimulation

We evaluated intracellular ROS produced by H_2O_2 stimulation to clarify the antioxidative properties of AsANa and APM. Intracellular ROS levels in the control group increased more than twofold due to H_2O_2 stimulation at all incubation times ($p < 0.001$). The intracellular ROS scavenging activity levels as a percentage of those of the H_2O_2 alone group were 35 (12 h; $p < 0.01$), 38 (24 h; $p < 0.001$), 35 (48 h; $p < 0.001$) and 16% (72 h; $p < 0.05$) in the APM group and 23 (12 h; $p < 0.05$), 18 (24 h; $p < 0.01$), −3 (48 h; not significant) and −10% (72 h; not significant) in the AsANa group (Fig. 3A). The APM group showed significant suppression of intracellular ROS production up to 72 h, whereas in the AsANa group, the suppressive effect on ROS production disappeared at 48 h. Figure 4 illustrates the cell images produced by transmitted or fluorescent light and quantification of fluorescence images when the cells were incubated for 24 h followed by H_2O_2 stimulation. The strength of the yellowish-green intracellular fluorescence, which corresponded to intracellular ROS levels, in the APM group was remarkably reduced compared with that in the H_2O_2 alone and AsANa groups. This was consistent with the findings in Fig. 3A. We also measured cell viability to clarify whether cell damage was influenced by this ROS suppression. In all groups, cell damage appeared 1 h

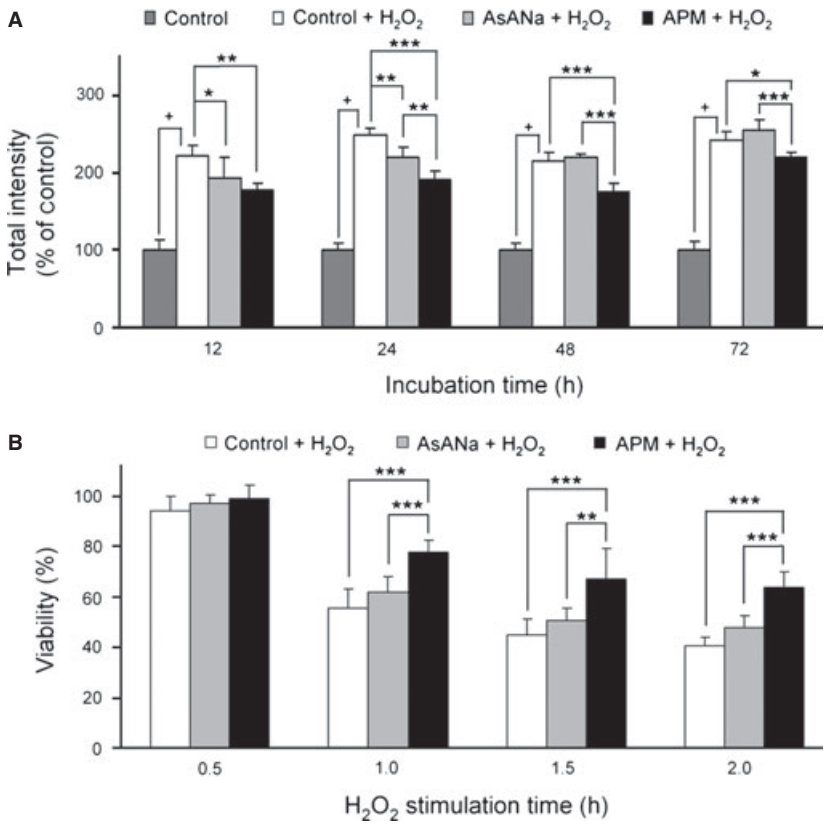

Fig. 3. Effects of APM and AsANa on hydrogen peroxide (H_2O_2)-induced intracellular reactive oxygen species (ROS) generation and cell damage in human gingival fibroblasts. Human gingival fibroblasts were incubated in the presence or absence (control) of 50 μM AsANa or APM in DMEM containing 1% FCS. (A) After incubation for 12–72 h, the cells were incubated for 30 min in serum-free DMEM containing 10 μM 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) and transferred to 1 mM H_2O_2 in serum-free DMEM. The ROS levels were immediately detected using a microplate fluorometer for 30 min. The results are shown as the means + SD ($n = 6$) of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Tukey's test; and +$p < 0.001$ by Unpaired student's *t*-test. (B) After a 24 h pretreatment for APM and AsANa, the cells were stimulated with 1 mM H_2O_2 for 0.5–2 h in serum-free DMEM and subsequently incubated for 30 min in Dulbeccos phosphate-buffered saline containing 1 μM calcein acetoxymethyl ester. The results are shown as the means + SD ($n = 6$) of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Tukey's test.

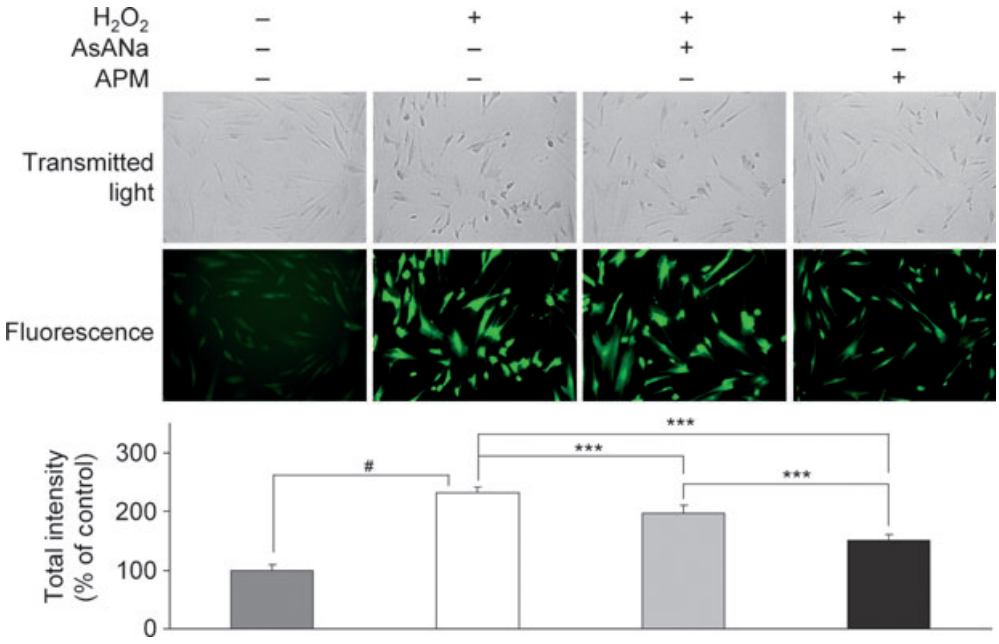

Fig. 4. Intracellular ROS scavenging activity of APM in human gingival fibroblasts. Human gingival fibroblasts were incubated in the presence or absence (control) of 50 μM AsANa or APM in DMEM containing 1% FCS for 24 h. After incubation, the cells were incubated for 30 min in serum-free DMEM containing 10 μM CM-H_2DCFDA and transferred to 1 mM H_2O_2 in serum-free DMEM for 30 min. Cell images were taken, and the fluorescence intensity of each image was quantified by the IN Cell Analyzer 1000. Cell images are representative of three independent experiments. Data are shown as the means + SD ($n = 6$) of three independent experiments. ***$p < 0.001$ by Tukey's test; and $p < 0.01$ by Aspin–Welch test.

after stimulation with H_2O_2, but cell damage in the APM group was significantly lower than that in the H_2O_2 alone ($p < 0.001$) and AsANa groups ($p < 0.01$; Fig. 3B).

APM inhibited IL-8 production by suppressing intracellular ROS through TNF-α stimulation

We investigated the effects of AsANa and APM on intracellular ROS and IL-8 production induced by TNF-α stimulation. The intracellular ROS level in the control group increased by approximately 40% due to TNF-α stimulation ($p < 0.001$). The APM group showed a 66% suppression of intracellular ROS levels ($p < 0.01$), but the AsANa group showed only an 8% suppression (not significant) in comparison to the values for the TNF-α alone group (Fig. 5A). In contrast, IL-8 production and mRNA expression increased remarkably after TNF-α stimulation. Interestingly, the APM group showed a 35% inhibition of IL-8 protein production ($p < 0.001$) and a 52% inhibition of IL-8 mRNA expression ($p < 0.01$) compared with the production in the TNF-α alone group (Fig. 5B and 5C). However, no significant differences were observed between the TNF-α alone and AsANa groups.

Discussion

APM is a stable AsA derivative, but the mechanism by which APM and AsA are taken up by human gingival fibroblasts has not yet been clarified. Interestingly, in our experiment quantifying intracellular AsA levels, the APM group showed an approximately 20-fold enhancement in the amount of AsA relative to that of the AsA group 24 h after incubation, and this effect was long lasting. We believe that enhancement and persistence of intracellular AsA in the APM group could be explained by the existence of AsA transporters and the gradual degradation of APM. Transport studies have revealed that mammalian cells are endowed with two types of transporters (SVCT 1 and SVCT 2) across their membrane (26), and various cells kinetically control the intake of AsA through these transporters by sodium or other cations (27,28). The transporters have a high specificity for AsA, but not for APM (26,29). The amount of intracellular intact APM in the APM group was lower than that of intracellular AsA, although we could detect intracellular APM. Therefore, we consider that intracellular AsA in the APM group was due to the intake of AsA after degradation. This possibility is supported by the result of the APM (inactivated) group wherein the amount of intracellular AsA was markedly decreased because of phosphatase inactivation in FCS. The lower amount of intracellular AsA in the APM (inactivated) group was probably due to active phosphatase on the cell membrane (30). Chepda *et al*. (30) also demonstrated that APM is gradually degraded to AsA to continuously supply AsA to the culture medium for many hours. This highly effective characteristic of APM has been demonstrated in other cells as well (31).

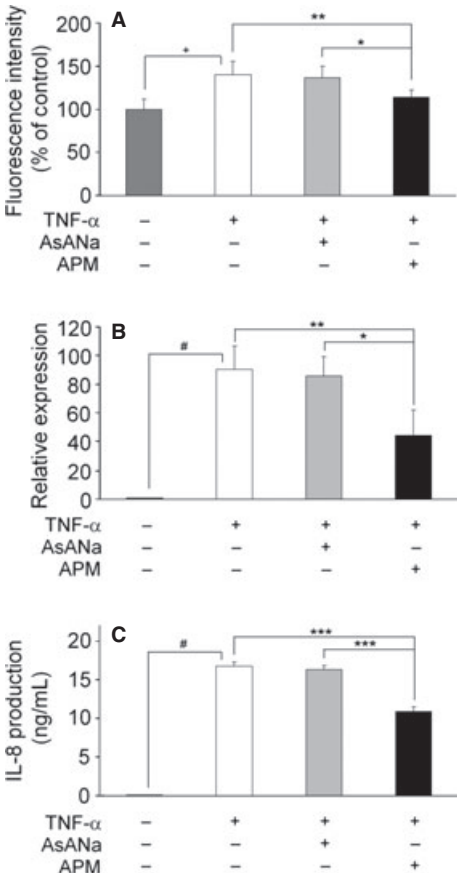

Fig. 5. Effects of APM and AsANa on tumor necrosis factor-α (TNF-α)-induced interleukin-8 (IL-8) production and intracellular ROS generation in human gingival fibroblasts. Human gingival fibroblasts were incubated in the presence or absence (control) of 50 μM AsANa or APM in DMEM containing 1% FCS for 24 h. After incubation, the cells were stimulated by 1 nM TNF-α for 8 h. (A) Intracellular ROS generation in human gingival fibroblasts was determined using CM-H_2DCFDA as described in the Material and methods section. Data are shown as the means + SD ($n = 6$) of three independent experiments. (B) Interleukin-8 mRNA expression was measured by real-time RT-PCR. The results are shown as the means + SD ($n = 4$) of three independent experiments. (C) Interleukin-8 in the culture medium was detected using an ELISA kit. The results are shown as the means + SD ($n = 6$) of three independent experiments. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ by Tukey's test; and $p < 0.01$ by Aspin–Welch test.

The main finding of this study was that type 1 collagen synthesis and intracellular ROS suppression were related to the amount of intracellular AsA. The type 1 collagen molecule consists of two α1 (1) and one α2 (1) polypeptide chains with a spiral form (32). Many studies have reported that AsA plays an important role as a cofactor for proline and lysine hydroxylase, which is required for the numerous hydrogen bonds needed to create the stable spiral form and produce a mature collagen structure (33). In the results shown in Fig. 3A, the promotion of collagen in the AsANa and APM groups at 24 h can be considered to be dependent on collagen maturation induced by increased intracellular AsA, because collagen mRNA expression did not change up to 24 h in the three groups, and collagen synthesis in the AsANa group after 48 h stopped at the same time that the intracellular AsA supply was exhausted. However, collagen synthesis after 48 h was accelerated in the APM group, contrary to the tendency of intracellular AsA levels to decrease. We would like to suggest that the increase in collagen mRNA expression strongly contributed to the acceleration in the APM group. Many studies have found that the type 1 collagen mRNA expression in various cells clearly increased in the presence of AsA, and this phenomenon has been attributed to mechanisms such as transcriptional control, a feedback effect produced by the procollagen polypeptide (34–36). In contrast, an increase in collagen mRNA expression in the AsANa group was not observed in this study. We speculate that this was because of the persistence of intracellular AsA at a high concentration. That is, in the present study, we only employed a single AsA treatment for 72 h, whereas daily AsA treatment was performed in most other studies.

Excess ROS levels have been implicated as the cause of various diseases, including periodontal disease (17). In periodontal lesions, neutrophils release excessive levels of superoxide, which is immediately converted to H_2O_2 (5). The H_2O_2 subsequently converts to hydroxyl radicals in the presence of metal ions in various tissues, and hydroxyl radicals induce the tissue dysfunction involved in cell damage (37). Previous studies have reported that H_2O_2 increases intracellular ROS and induces damage in various cells (17,21). In our study, the APM group showed remarkable suppression of intracellular ROS production and decreased cell damage after H_2O_2 stimulation, similar to the findings for other antioxidants (38,39). In addition, the effect was related to the amount of intracellular AsA that was capable of scavenging ROS (17). Thus, APM, which greatly enhanced intracellular AsA levels, can be expected to prevent tissue destruction caused by ROS in the progression of periodontal disease.

In this study, we investigated the anti-inflammatory properties of APM against the stimulatory effects of TNF-α, which is a major proinflammatory cytokine and induces intracellular ROS as signaling intermediates for proinflammatory cytokine production (17,24,40). On TNF-α stimulation, APM suppressed intracellular ROS production and inhibited IL-8 production in human gingival fibroblasts. These

effects appeared to be related to the intracellular AsA content. Many studies have reported that IL-8, which is an important factor in the chemotaxis of neutrophils (41), is induced by TNF-α stimulation in various cell types, including human gingival fibroblasts (42,43), and is detected in periodontal lesions (44,45). *N*-Acetyl-L-cysteine, a representative antioxidant, inhibited TNF-α-induced IL-8 production through the suppression of intracellular ROS (24). Furthermore, O'Hara *et al.* (46) found that *N*-acetyl-L-cysteine inactivated redox-sensitive transcription factors, such as nuclear factor-κB and activator protein-1. We speculate that the suppression of intracellular ROS production by APM may contribute to the inhibition of IL-8 production through the inactivation of nuclear factor-κB and activator protein-1 in human gingival fibroblasts.

Periodontal disease is an inflammatory disorder caused by periodontal pathogens. During the inflammatory process, MMP and ROS cause the destruction of periodontal tissues (5). Furthermore, continuous and excessive IL-8 secretion by human gingival fibroblasts induces unduly chemotactic activity of neutrophils and the initiation of chronic inflammatory responses, eventually leading to tissue destruction (41,44,45). Hence, the topical application of APM to periodontal lesions may promote gingival collagen synthesis and suppress excess ROS production through the enhancement of intracellular AsA levels, thereby blocking the destruction of periodontal tissue. A previous study reported that the phosphate derivatives of AsA penetrated through the epidermis of hairless mouse skin and were converted to AsA by phosphatase in the cell membrane (13). In addition, Shibayama *et al.* (47) demonstrated that the phosphate derivatives of AsA penetrated through a human epidermal skin model consisting of keratinocytes rather than AsA, after which they reached the site of the dermis mainly consisting of fibroblasts and collagen. Therefore, APM can be judged to penetrate through the gingival epithelia and reach the gingival fibroblasts, where it subsequently promotes gingival collagen synthesis, suppresses excess ROS production by enhancing intracellular AsA, and regulates the chronic inflammatory response through ROS suppression. Thus, the local application of APM can prevent periodontal disease.

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