Effect of a Novel Ascorbic Derivative, Disodium Isostearyl 2-O-L-Ascorbyl Phosphate, on Normal Human Dermal Fibroblasts against Reactive Oxygen Species

Hiroharu SHIBAYAMA,† Masayoshi HISAMA,‡ Sanae MATSUDA,‡ Atsushi KAWASE,§ Mamitaro OHTSUKI,∥ Katsumi HANADA,¶ and Masahiro IWAKI

1Department of Dermatography, Jichi Medical University, 3311-1 Yakushiji, Shimotsuke, Tochigi 329-0498, Japan
2Central Research Center, Toyo Beauty Co., Ltd., 3-13-8 Higashinakamoto, Higashinari-ku, Osaka 537-0021, Japan
3Department of Pharmacy, School of Pharmacy, Kinki University, 3-4-1 Kowakae, Higashi-osaka, Osaka 577-8502, Japan
4Department of Dermatology, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8562, Japan

Received November 26, 2007; Accepted January 10, 2008; Online Publication, April 7, 2008

The novel amphiphilic vitamin C derivative disodium isostearyl 2-O-L-ascorbyl phosphate (VCP-IS-2Na), which has a C18 alkyl chain attached to the stable ascorbate derivative sodium L-ascorbic acid 2-phosphate (VCP-Na), was evaluated for reduction of cell damage induced by oxidative stress, ultraviolet A (UVA), ultraviolet B (UVB), and H2O2; stimulation of collagen synthesis against UVA irradiation; and inhibition of matrix metalloproteinase-1 (MMP-1) activity induced by UVA in human normal dermal fibroblasts. VCP-IS-2Na pretreatment resulted in significant protection against cell damage induced by UVB, UVA, and H2O2. The amount of type I collagen following UVA irradiation was increased by treatment with VCP-IS-2Na in a concentration-dependent manner. These effects of VCP-IS-2Na were superior to those of L-ascorbic acid (vitamin C, VC) and VCP-Na. On the other hand, VCP-IS-2Na suppressed 65% of the excess MMP-1 irradiated UVA, and VC and VCP-Na slightly suppressed it.

Key words: amphiphilic vitamin C derivative; oxidative stress; disodium isostearyl 2-O-L-ascorbyl phosphate; human skin fibroblast; VCP-IS-2Na

Aging of skin is a complex biological phenomenon consisting of two components: intrinsic aging and photoaging caused by environmental exposure, primarily to ultraviolet (UV) light, but decreased metabolic function and accumulated oxidative damage induced by reactive oxygen species (ROS) are responsible for cutaneous inflammatory disorders and skin aging.1,2)

Wrinkling of the skin, including dryness, roughness, and pigmentation, is a common phenomenon in aged skin. Dermal-epidermal junctions are also weaker. Striking changes are observed in the dermis, in which there is a loss of thickness. There is a decrease in the number of elastic fibers and in skin elasticity. These phenomena are accompanied by a decrease in dermal cell activity, including a decrease in fibroblast proliferation and low cellular communication by cytokines. Aged skin especially shows a decrease in collagen production. In the case of photoaging, the collagen content is decreased, because of increased degradation and decreased synthesis. Decreased collagen affects the dermal architecture and the stretching and elasticity of skin, which causes fine wrinkling.3)

Normal human dermis consists primarily of an extracellular matrix (ECM) of connective tissue. Three major extracellular components have been recognized that contribute to the physiological properties of the skin. Specifically, fibers consisting of collagen, an abundant ECM protein that accounts for about 80% of the dry weight of the skin, provide tensile properties to the dermis, so as to allow the skin to serve as a protective organ against external trauma.4) The elastic fibers, which account for 2–4% of the ECM in sun-protected skin, form an interconnecting network that provides elasticity and resilience to normal skin.5) The ECM serves not only as a scaffolding to stabilize tissue structure, but also has been observed to influence the development, migration, proliferation, shape, and metabolic function of cells with which it comes into contact. Thus processes that alter the relative proportions of

† To whom correspondence should be addressed. Tel: +81-6-6971-0271; Fax: +81-6-6971-1631; E-mail: UEB37453@nifty.com
ECM can result in clinical manifestations that are recognized as part of the cutaneous aging process.

More recently, changes in collagen metabolism have been brought into focus as a major factor leading to photoaging. Specifically, it has been found that accumulation of elastic material is accompanied by concomitant degeneration of the surrounding collagenous meshwork, and evidence implicating matrix metalloproteinases (MMPs) as mediators of collagen damage in photoaging has been presented.\textsuperscript{5-7} MMPs comprise a family of degradative enzymes consisting of at least 14 different members with rather broad substrate specificity.\textsuperscript{8} Many of these proteases can degrade native collagen fibers, denatured collagen, elastic fibers, various proteoglycans, and fibronectin, among other components of the dermis. It has been demonstrated in vitro that UV irradiation of fibroblasts in culture enhances the expression of these proteolytic enzymes.\textsuperscript{9,10}

Vitamin C (\(\alpha\)-l-ascorbic acid) functions in many biological processes, such as collagen synthesis, antioxidant, intestinal adsorption of iron, and metabolism of some amino acids.\textsuperscript{11,12} Vitamin C is an ROS scavenger, and it increases collagen synthesis in dermal fibroblasts due to its functioning as a co-factor of 4-hydroxyproline synthesis.\textsuperscript{13} In in vitro studies, it has been demonstrated that vitamin C prevents the formation of skin wrinkling.\textsuperscript{14} On account of these functions, vitamin C has been accepted as an attractive anti-aging agent. However, it is difficult to determine the effects of vitamin C in the skin, because it is less resistant to oxidative conditions than other vitamins and is easily degraded. Vitamin C efficiently scavenges ROS in aqueous solutions, while it has difficulty scavenging ROS in lipid layers, such as lipoproteins and the cell membrane. We recently synthesized a novel lipophilic alkyl vitamin C derivative, sodium isostearyl 2-\(\alpha\)-ascorbyl phosphate (VCP-IS-Na), which is characterized by its high stability in various aqueous solutions over a wide range of \(pH\) values, and its non-reducibility.\textsuperscript{15} We also found that VCP-IS-Na is superior to the stable ascorbate derivative sodium \(\alpha\)-ascorbic acid 2-phosphate (VCP-2Na) with regard to enzymatic hydrolysis by tissue esterase and/or phosphatase to give vitamin C. These findings imply that vitamin C, released from VCP-IS-Na by enzymatic hydrolysis, exhibits these biological activities.

In the present study, we found that VCP-IS-2Na, which increased stability on exposure to alkali, oxidation, and prolonged storage more than VCP-IS-Na, effectively reduced cell damage induced by physical (UVA and UVB) and chemical (\(H_2\)O\(_2\)) oxidative stresses, suppressed the UVA-induced decrease in type I collagen, and inhibited matrix metalloproteinase-1 (MMP-1) production induced by UVA irradiation in normal human dermal fibroblasts. An increase in vitamin C, which is metabolized from VCP-IS-2Na, in normal human dermal fibroblasts was also found. The aim of this study was to clarify the potential of VCP-IS-2Na for prevention of the effects of aging and wrinkle formation. Recent advances in medical assistance and equipment have resulted in a worldwide increase in the elderly population. This tendency has given rise to a growing demand for effective skin-care agents and supplements to maintain health and beauty. We found in this study that VCP-IS-2Na prevented wrinkle formation with direct application to skin by its excellent anti-oxidative properties.

Materials and Methods

General procedures. \(^1H\text{- and }^{13}C\text{-NMR spectra (\(\delta\), \(J\) in Hz) were recorded on a JEOL GSX 500 NMR spectrometer. Tetramethylsilane was used as the internal reference (\(\delta\) 0.00) for \(^1H\text{-NMR spectra measured in chloroform-d}_4\). This solvent was also used for \(^{13}C\text{-NMR spectra. Infrared (IR) spectra were determined with an FT/IR-470 Pulse Fourier Transform Infrared Spectrometer using a KBr disk. Fast atom bombardment high resolution mass spectra (FAB-HR-MS) and FAB-MS spectra were obtained on a JEOL JMS-HX 100 mass spectrometer. UV spectra were obtained on Shimadzu UV-2450 spectrophotometer. Optical rotations were measured with JASCO DIP-1000. HPLC analysis was carried out with a system consisting of a Shimadzu SCL-10AVP system controller, LC-10ADVP pump, SPD-10AVP \textit{UV–Vis} spectrophotometric detector, SIL-10APV auto-injector, CTO-10A column oven, and CLASS-VP chromatopac.

Chemicals. \(\alpha\)-Ascorbic acid was obtained from Sigma–Aldrich (St. Louis, MO). VCP-Na salt and MTT (3-(4,5-dimethylthiazol-2,5-diphenyl-tetrazolium bromide) were obtained from Wako Pure Chemical Industries (Osaka, Japan). Isostearyl alcohol was obtained from Nissan Chemical Industries (Tokyo). Phosphatase inhibitor cocktail (PIC) II was obtained from Merck (Tokyo).

Synthesis of VCP-IS-2Na. Isostearyl 2-\(\alpha\)-vasorubin phosphate (VCP-IS) was synthesized from 5,6-\(\alpha\)-isopropylidene-\(\alpha\)-ascorbic acid and isostearyl dichlorophosphate using our previously reported methods.\textsuperscript{16} VCP-IS (1 mol) was reacted with sodium hydrate (2 mol). The reaction mixture was stirred at 0 \(^\circ\)C for 3 h and ethanol was added and evaporated in vacuo. The residue was subjected to recrystallization in acetonitrile and ethanol to give VCP-IS-2Na (65% yield) as a white powder. For VCP-IS-2Na: \(^1H\text{-NMR (CDCl}_3\): \(\delta\) 0.88 (24H, \(t\), \(J = 7.3\), CH3-11, 13, 15, 17), 1.00–1.05 (2H, m, 12-H), 1.1–1.19 (2H, m, 16-H), 1.21–1.25 (4H, m, 9, 10-H), 1.26–1.33 (1H, m, 11-H), 1.47–1.53 (1H, m, 15-H), 1.76–1.82 (1H, m, 8-H), 3.81 (2H, m, 7-H), 3.95 (2H, d, \(J = 7.0\), 6-H), 4.12 (1H, dd, \(J = 1.8, 7.0\), CH5), 4.81 (1H, d, \(J = 1.8\), 4-H), 13C-NMR: 171.18 (C-1), 167.63 (C-3), 110.58 (C-2), 77.41 (C-4), 69.89 (C-7), 69.04 (C-5), 63.25 (C-6), 51.23 (C-12), 48.56 (C-16), 45.98 (C-8), 38.03 (C-10), 31.21 (C-13), 31.05 (C-14), 30.90 (C-14),
Effect of a Novel Ascorbic Derivative, Disodium Isostearyl 2-O-t-Ascorbyl Phosphate

Fig. 1. Structure of VCP-IS-2Na.

Cell culture. Normal human dermal fibroblasts (NHDFs) were purchased from Kurabo (Osaka), and cultured with Kurabo’s modified Medium 106S containing 5% fetal bovine serum (FBS). The fibroblasts were assayed for reduction of cell damage, collagen, and collagenase synthetic activity, as described below.

HPLC conditions. Separation of vitamin C was achieved by isocratic elution from an RP-18 (4.6 × 250 mm, 5 μm; GL Science, Tokyo) kept at 40°C, with 0.1 M sodium acetate/acetic acid buffer (pH 5.0) containing 4% methanol, 2.8% n-hexylamine, and 0.01% disodium edentate, at a flow rate of 0.7 ml/min. Spectrum absorbance at 245 nm was monitored.

Reduction of cell damage induced by oxidative stress. NHDF cells were inoculated in 96-well plates at a density of 2.0 × 10^4 cells/well. After 24 h pretreatment with VCP-IS-2Na at several concentrations, cells were exposed to UVB (30 mJ/cm^2) or UVA (15 J/cm^2) in Hank’s buffered solution (HBS). The viability of the cells was evaluated by MTT assay after 24 h of cultivation. The MTT assay was based on the Mosmann method. In the case of H_2O_2 exposure, cell viability was estimated by MTT assay at 2 h after exposure to H_2O_2 (2 mM). To demonstrate that the reductive effect of VCP-IS-2Na on cell damage induced by oxidative stress was caused, not by VCP-IS-2Na but by vitamin C, which is produced by hydrolysis with phosphatase in the fibroblasts, NHDF cells were cultured with Medium 106S or HBS containing the PIC II at several concentrations.

Amount of vitamin C in human skin fibroblasts. NHDF cells were inoculated into 75-cm^2 flasks (1.0 × 10^6 cells/flask) and cultivated for 24 h. After culture, the medium was changed to serum-free medium containing 50 μM VCP-IS-2Na and cultivated for 24 h. After a fixed period of time, cultured NHDF cells from 10 flasks were washed 3 times with PBS, and 1.0 ml of PBS was added. The cells were then scraped out with a cell scraper and transferred to an Eppendorf tube. The cell suspension (1.0 × 10^7 cells/ml) in 0.1 M PBS was sonicated and centrifuged at 4,000 rpm (4°C) for 10 min to obtain a supernatant. The supernatant was analyzed by HPLC. To confirm that VCP-IS-2Na produced VC by hydrolysis with phosphatase existing in the fibroblasts demonstrate the effects with phosphatase inhibitor, it was determined whether NHDF cells were cultured with medium containing 0.1% PIC II.

Statistical analysis. The proliferation, collagen content, MMP-1 activity, and amount of vitamin C data were expressed as the mean ± standard error (S.E.), and subsequent inspection of means was evaluated by Student’s t-test between two groups at significance levels of p < 0.05 and p < 0.01.

Results

Reduction of cell damage induced by oxidative stress
Pretreatment of NHDF cells with VCP-IS-2Na significantly protected against cell damage induced by UVB, UVA, and H_2O_2, in a dose-dependent manner (Fig. 2). VCP-IS-2Na at 50 μM showed 35, 29, and 24% protection against cell damage induced by UVB, UVA and H_2O_2 respectively. VCP-Na showed 14, 12, and 11% protection, and vitamin C showed very little protection, at the same concentrations. These protective effects of VCP-IS-2Na against cell damage induced by UVB, UVA, and H_2O_2 were superior to those of vitamin C and VCP-Na. In the presence of the phosphatase inhibitor, VCP-IS-2Na and VCP-Na showed decreased protection against cell damage induced by UVB, UVA,
and \( \text{H}_2\text{O}_2 \) in the presence of phosphatase inhibitor (Table 1). In the presence of 0.10% PIC II, the protective effect of VCP-IS-2Na at 50 \( \mu \text{M} \) against cell damage induced by UVB, UVA, and \( \text{H}_2\text{O}_2 \) decreased by 24, 22, and 17% respectively. The protective effect of VCP-Na decreased by 14, 10, and 11%, respectively, and the effect of vitamin C did not show any decrease.

**Suppression of UVA-induced decrease in collagen**

Pretreatment of NHDF cells with VCP-IS-2Na showed significant suppression of the decrease in collagen induced by UVA irradiation, in a dose-dependent manner (Fig. 3). VCP-IS-2Na at 50 \( \mu \text{M} \) significantly decreased the UVA-irradiation-induced decrease in collagen, by 42%. Vitamin C and VCP-Na showed 17 and 14% suppression respectively, at the same concen-
Suppression of UVA-induced production of MMP-1

Pretreatment of NHDF cells with VCP-IS-2Na showed significant suppression of the excess production of MMP-1 induced by UVA irradiation, in a dose-dependent manner (Fig. 3). VCP-IS-2Na at 50 μM showed a significant 65% suppression of the excess production of MMP-1 induced by UVA irradiation. On the other hand, vitamin C and VCP-Na showed only 10 and 14% suppression respectively at the same concentration. In the presence of phosphatase inhibitor, the suppressive effect of VCP-IS-2Na and VCP-Na against the excess production of MMP-1 induced by UVA irradiation decreased, in a manner independent of the concentration of phosphatase inhibitor (Fig. 4). PIC II at 0.10% decreased by 81% the suppressive effect of VCP-IS-2Na against excess production of MMP-1 after UVA irradiation. The same concentration of PIC II decreased the suppressive effect by 99%, but it did not affect the suppressive effect of vitamin C.

Vitamin C content in normal human skin fibroblasts

We measured the effect of VCP-IS-2Na on vitamin C content in human skin fibroblasts. In NHDF cells treated with VCP-IS-2Na, the amount of vitamin C was 59 nmol after 24h incubation (Fig. 5). In cells treated with vitamin C or VCP-Na, the amount of vitamin C in fibroblasts was 28 and 20 nmol respectively after 24h of incubation. In cells treated with VCP-IS-2Na and VCP-Na, phosphatase inhibitor decreased the amount of vitamin C. PIC II at 0.10% decreased the amount of vitamin C metabolized from VCP-IS-2Na in NHDFs by 77%. The same concentration of PIC II decreased the amount of vitamin C metabolized from VCP-Na by 58%, but it did not affect the amount of vitamin C present in NHDFs.

Discussion

In the present study, the reductive effect of cell-damage induced physical and chemical reactive oxygen species (ROS) and the suppressive effect of the decrease in collagen and MMP-1 production induced by UVA irradiation were examined in normal human dermal fibroblasts culture system to address the anti-aging effects of VCP-IS-2Na in biological systems. Pretreatment of normal human dermal fibroblasts with VCP-IS-2Na led to significant protection against cell damage induced by physical (UVB, UVA) and chemical (H₂O₂)
ROS (Fig. 2). It has been established that UVB produces superoxide anion radicals mainly by stimulation of the mitochondrial respiratory chain reaction.16) Superoxide anion radicals spontaneously convert to \( \text{H}_2\text{O}_2 \) and then to hydroxyl radicals in the presence of metal ions, Fe\(^{2+}\), and Cu\(^{+}\), As17) The substance involved in cell damage during \( \text{H}_2\text{O}_2 \) exposure was the hydroxyl radical. Based on biological and chemical test results after exposure to UVB, \( \text{H}_2\text{O}_2 \), or hydroxyl radicals, we suggest that VCP-IS-2Na was converted to its reduced form after uptake into the cells. Vitamin C is the most important water-soluble antioxidant in human plasma,18) because its scavenging spectrum for ROS is extensive. It scavenges superoxide anions, hydroxyl radicals, lipid peroxyl radicals and singlet oxygen.19,20) Our results indicate that the presence of phosphatase inhibitor decreased the protective effect of VCP-IS-2Na against cell damage induced by ROS (Table 1). Hence, the anti-oxidative effects of VCP-IS-2Na originate from the metabolized form vitamin C, which is produced by tissue esterase and/or phosphatase in normal human dermal fibroblasts.

It is acknowledged that alteration in the quantity and character of collagen is a critical factor in the process of wrinkle formation. UV (UVB and UVA) irradiation of the skin causes changes in the metabolism of collagen matrix. UVA irradiated-fibroblasts have been found to show decreased collagen production and increased MMP-1 production.21) Repetition of these reactions causes a significant decrease in dermal collagen, leading to the formation of very fragile dermis and wrinkles. VCP-IS-2Na significantly suppressed the excess decrease in type I collagen and the production of MMP-1 following UVA irradiation in normal human dermal fibroblasts (Fig. 3). It has been established that singlet oxygen produced in cells by UVA irradiation up-regulates MMP-1 gene transcription.22) Also, VCP-IS-2Na reduced the damage to normal human dermal fibroblasts exposed to UVA. These results suggest that VCP-IS-2Na suppressed MMP-1 production by quenching singlet oxygen, superoxide, and free radicals, and thus suppressed the decrease in type I collagen caused by inhibition of MMP-1 production and the effect of vitamin C, which is metabolized from VCP-IS-2Na and decreased these anti-aging effects of VCP-Na and VCP-IS-2Na in terms of all anti-aging effects, protection against cell damage induced by physical and chemical oxidative stress, suppressing the excess decrease in type I collagen induced by UVA irradiation, and inhibition of excess MMP-1 production induced by UVA irradiation in normal human fibroblasts. Phosphatase inhibitor decreased the suppressive effects of VCP-IS-2Na against the decrease in type I collagen and MMP-1 production induced by UVA irradiation in normal human dermal fibroblasts (Fig. 4). Hence, the suppressive and inhibitory effects of VCP-IS-2Na originate from the metabolized vitamin C, which is produced by tissue esterase and/or phosphatase in normal human dermal fibroblasts. VCP-IS-2Na was superior to vitamin C and VCP-Na in terms of all anti-aging effects, protection against cell damage induced by physical and chemical oxidative stress, suppressing the excess decrease in type I collagen induced by UVA irradiation, and inhibition of excess MMP-1 production induced by UVA irradiation in normal human fibroblasts. Phosphatase inhibitor decreased these anti-aging effects of VCP-Na and VCP-IS-2Na in a dose-dependent manner, although the effects of vitamin C were not decreased. The rigid lipid lamella structure of the stratum works as a barrier, especially to the adsorption of hydrophilic compounds.23) We found that the amount of vitamin C in normal human fibroblasts after 24h of incubation with VCP-IS-2Na was much greater than in the presence of vitamin C or VCP-Na (Fig. 5). These results suggest that the anti-aging effects of the novel amphiphilic vitamin C derivative VCP-IS-2Na were due to its permeation and metabolism superior to that of vitamin C, in normal human fibroblasts.
Conclusion

We synthesized the novel amphiphilic vitamin C derivative VCP-IS-2Na, which improved stability more than sodium isostearyl 2-O-L-ascorbyl phosphate. It is accepted that endogenous and exogenous oxidative stress is a critical factor in the aging process. VCP-IS-2Na showed protective effects against physical and chemical oxidative stress, and a suppressive effect against excess type I collagen decrease and MMP-1 production induced by UVA irradiation with released VC through hydrolysis of esterase and/or phosphatase in normal human fibroblasts. These effects of VCP-IS-2Na were superior to those of vitamin C and VCP-Na due to the excellent intercellular VC activity of VCP-IS-2Na. Type I collagen and MMP-1 play a role in the process of wrinkle formation, which is a common phenomenon of aged skin. These findings indicate that VCP-IS-2Na was an effective agent for the supply of vitamin C with transdermal activity in skin care. In conclusion, we expect that VCP-IS-2Na with such characteristic properties will be utilized not only in the field of cosmetics, but also in other fields.

References

13) Lyons, B. L., and Schwarz, R. I., Ascorbate stimulation of PAT cells causes an increase in transcription rates and


