LIPID-SOLUBLE VC DERIVATIVE ASCORBIC ACID TETRA-2-HEXYLDECANOATE (VC-IP) AS AN ANTI-AGING AGENT

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Synopsis

It is well known that ascorbic acid (VC) is an important factor for several physiological reactions. In the skin, VC works as an anti-aging agent due to removing of oxidative stress generated by UV irradiation and stimulation of collagen synthesis. Thus, developing more effective VC derivatives is an important issue in creating anti-aging skin care products. Our study succeeded to develop a novel ascorbic acid derivative, ascorbic acid tetra-2-hexyldecanoate (VC-IP), which is a lipid-soluble pro-VC. The purpose of this study was to indicate the effects of VC-IP as pro-VC and anti-aging agent. First, it was examined whether VC-IP is converted to VC in physiological conditions. Since VC was detected from the cell extracts treated with VC-IP, it was indicated that VC-IP is a pro-VC. At the next step, the efficacy of VC-IP as an anti-aging agent was evaluated. It has been authorized that reactive oxygen species (ROS) generated by UV irradiation are responsible for acceleration of skin aging, which is characterized by wrinkles and pigment spots. Therefore, VC-IP efficacy in reducing oxidative stress was examined. It was found that VC-IP reduced cell damage, which is induced by UVB, H_2O_2 and *t*-BuOOH, and DNA damage after UVB irradiation. In addition, the topical application of VC-IP on the skin decreased the generation of squalene peroxide by UVB irradiation. On the other hand, VC-IP increased type I collagen synthesis of human normal dermal fibroblasts as well as VC and decreased melanin synthesis of B16 melanoma cells. From these results, it was indicated that VC-IP possesses excellent features of anti-aging agent for improving wrinkles and pigment spots.

Introduction

It is widely accepted that wrinkle formation, sagging and pigment spots characterize facial skin aging, and reactive oxygen species (ROS) are responsible for skin aging. Therefore, it is estimated that ROS scavengers effectively work as anti-aging agents. The formation of ROS is prevented by an antioxidant system in human body such as low molecular mass antioxidants (ascorbic acid, glutathione, tocopherols) and ROS-scavenging enzymes (superoxide dismutase, glutathione peroxidase and catalase) (1).

Vitamin C (VC, ascorbic acid) is the most important water-soluble antioxidant in human plasma (2), because its scavenging spectrum on ROS is extensive. VC scavenges superoxide anion, hydroxyl radical, lipid peroxyl radical and singlet oxygen (3, 4). In *in vivo* studies, it is identified that VC shows the suppression of sunburn (5), delays the onset of skin tumors by UVB irradiation (6), and prevents the formation of skin wrinkling (7).

On the other hand, the decrease or alteration of collagen fiber is one of important factors of wrinkle formation (8,9). VC increases collagen synthesis of dermal fibroblasts due to its functioning as a co-factor of 4-hydroxyproline synthesis (10). In addition, VC expresses the whitening/lightening effects by inhibition of tyrosinase activity (11). On these functions, it is acceptable that VC is an attractive active agent for anti-aging.

However, it is hard to exhibit the effects of VC in the skin, because VC is rapidly oxidized in formulations. Therefore, to overcome the weak points of VC, several stabilized VC derivatives (water-soluble or lipid-soluble) have been synthesized. In this study, anti-aging efficacy of ascorbic acid tetra-2-hexyldecanoate (VC-IP, Fig.1), a newly synthesized lipid-soluble pro-vitamin C, was examined.

Materials and Methods

Conversion of VC-IP into VC

Mouse keratinocytes or human fibroblasts were treated with VC-IP or VC. After 2 h incubation, cells were homoginated and the content of free ascorbic acid were determined using HPLC.

Oxidative stress

HaCaT keratinocytes which were pre-treated with Dulbecco's modified Eagles medium (DMEM) containing 5% fetal bovine serum (FBS) and 100 μM VC-IP or another VC derivatives (VCNa, VCPMg) for 24h, were exposed to UVB, H_2O_2 or *t*-BuOOH. Cell viability was measured with neutral red (NR) assay.

UVB irradiation

Cells were applied to Hank's buffered solution (HBS) and then exposed to UVB at energy of 200 mJ/cm^2. Following UVB irradiation, cell viability was quantified using neutral red (NR) assay. The medium was replaced with DMEM containing 5% FBS and NR reagent and incubated for 2 h. Then cell viability was quantified by measurement of absorbance at 540nm.

H_2O_2

Cells were applied to HBS containing 20 mM H_2O_2 and incubated for 2 h. Following H_2O_2 treatment, cell viability was quantified using NR assay.

tert-butylhydroperoxide (*t*-BuOOH)

Cells were applied to HBS containing 1 mM *t*-BuOOH and incubated for 4 h. Following *t*-BuOOH treatment, cell viability was quantified using NR assay.

DNA damage

HaCaT cells were treated with DMEM containing 5% FBS and VC-IP for 24 h. Following UVB irradiation at energy of 15 mJ/cm^2, DNA damage were evaluated using single cell gel electrophoresis assay (comet assay) (12). Comet assay detects DNA damage, mainly DNA strand breaks and alkali labile sites in the DNA molecule.

Generation of squalene peroxide

10% VC-IP solution or placebo solution was applied on the inside of forearm of volunteers. After 4 h, UVB was irradiated to the applied forearm. Then, sebum was extracted. The amount of squalene and squalene peroxide were determined by HPLC analysis.

Type I collagen synthesis

Normal Human Dermal Fibroblast were treated with DMEM containing 5% FBS and VC-IP. After 24h incubation, the content of type I collagen in the medium were evaluated using ELISA method.

Melanogenesis

B16 melanoma 4A5 cells were treated with DMEM containing 5% FBS and various concentrations of VC-IP. After 4 days cultivation, cells were harvested by trypsinization and centrifuged to make cell pellets. Visualized score evaluated amounts of melanin of cell pellets.

Results and Discussion

VC-IP was converted to VC in cells

We examined the conversion of VC-IP to VC in physiological conditions. Amounts of intracellular VC in mouse keratinocytes and human fibroblasts, which were treated with VC-IP or VC for 2 h were quantified. In the case of mouse keratinocytes, intracellular VC of cells treated with 20 μM VC or VC-IP was 4 nmol/10^6cells or 11.7 nmol/10^6cells, responsively (Fig.2). Additionally, VC-IP-treated fibroblasts showed higher amount of intracellular VC than VC-treated fibroblasts. These results indicated that VC-IP was converted to VC after incorporating into the cells.

VC-IP reduced of cell damage induced by UVB, H_2O_2 and *t*-BuOOH

To investigate the anti-oxidation effect of VC-IP in biological tests, its protective effects against UVB, H_2O_2 and *t*-BuOOH were tested using HaCaT cells culture system.

When exposing cells to UVB, viability of cells decreased to 77.1 % of the sham-irradiated cells. It was found that the pretreatment of VC-IP at concentrations of 100 μM reduced the cell damage. On the other hand, VCNa and VCPMg failed to find the protection of the cell damage (Fig.3). The viability of the cells, which were exposed to H_2O_2, decreased to 38.2 % of the sham-exposed cells. The cell damage was reduced by the pretreatment of VC-IP at concentrations of 100 μM. On the other hand, VCNa and VCPMg failed to find the protection of the cell damage. The viability of the cells, which were exposed to *t*-BuOOH, decreased to 74.6% of the sham-exposed cells. The cell damage was reduced by the pretreatment of VC-IP at concentrations of 100 μM. On the other hand, VCNa and VCPMg failed to exhibit the protection of the cell damage.

From these results, it was suggested that VC-IP showed excellent protection against each type of oxidative stress: UVB, H_2O_2 and *t*-BuOOH.

VC-IP suppressed DNA damage after UVB irradiation

The effect of VC-IP on DNA damage was evaluated by comet assay. On comparing the length of comet tails, VC-IP pretreated cells showed shorter DNA tail than control cells (control, 80.2+/-11.2 μm; VC-IP treated cells, 59.9+/-6.7 μm) (Fig.4). The result suggested that VC-IP suppressed the DNA damage after exposure of UVB irradiation.

VC-IP decreased generation of squalene peroxide after UVB irradiation

The amounts of squalene peroxide on the inside of forearm of volunteers were increased by UVB irradiation. The squalene peroxides were reduced by the pretreatment of VC-IP. From this result, we suggested that VC-IP decreased peroxidation of squalene after exposure of UVB irradiation.

Anti aging effects

VC-IP increased synthesis of type I collagen

It was well known that VC increases the collagen synthesis. Therefore, the stimulative effect of VC-IP on collagen synthesis of human fibroblasts was evaluated by ELISA for type I collagen. The collagen protein level of cells, which were cultured with VC-IP at concentration of ranging from 10 μM to 50 μM, was increased in a dose dependent manner (Fig.5).

VC-IP suppressed the melanogenesis of B16 melanoma cells

We evaluated the inhibitory effects of VC-IP on melanogensis. As a result, the melanin contents in B16 melanoma cells treated with VC-IP decreased in a dose-dependent manner.

From these results, it was indicated that VC-IP increased the collagen synthesis of human dermal fibroblasts and suppressed the melanin production of B16 melanoma cells.

Conclusion

It is acceptable that endogenous or exogenous oxidative stress is a critical factor in advancing aging process. In this study, it was demonstrated that VC-IP showed excellent ability on reducing exogenous oxidative stress. Furthermore, VC-IP increased the collagen synthesis of human dermal fibroblasts and suppressed the melanin production of B16 melanoma cells. These results elucidated that VC-IP worked on both prevention and improvement of skin aging due to its abilities of oxidative stress reduction, enhancement of collagen synthesis and suppression of melanin production.

It is concluded that VC-IP, a novel type lipid-soluble pro-vitamin C, can be effectively used in various cosmetic applications as an anti-aging active ingredient.

References

1. Frei B., England L. and Ames B. N. (1989) Proc. Natl. Acad. Sci. U. S. A. **86**, 6377-81
2. Som S., Raha C. and Chatterjee I. B. (1983) Acta Vitaminol. Enzymol. **5**, 243-50
3. Nishikimi M. (1975) Biochem. Biophys. Res. Commun. **17**, 463-8
4. Retsky K. L., Freeman M. W. and Frei B. (1993) J. Biol. Chem. **268**, 1304-9
5. Darr D. et al. (1992) Br. J Dermatol. **127**, 247-53

6. Black H.S. (1987) Photochem. Photomed. **46**, 213-21

7. Bissett D.L, Chatterjee R. and Hannon D.P. (1990) Photodermatol. Photoimmunol. Photomed. **7**, 56-62.

8. Plastow S.R., Lovell C.R. and Young A.R. (1987) J Invest. Dermatol. **88**, 145-8

9. Kligman L.H., Gebre M. (1991) Photochem. Photobiol. **54**, 233-7

10. Lyons B.L., Schwarz R.I. (1984) Nucleic Acids Res. **12**, 2569-79

11. Lindbladh C., Rorsman H., and Rosengren E. (1983) Acta. Derm. Venereol. **63**, 209-14

12. Singh N.P. et al. (1988) Exp. Cell Res. **175**, 184-91

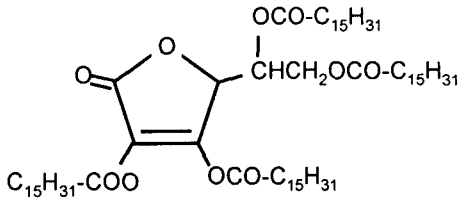

Fig.1 Structure of ascorbic acid tetra-2-hexyldecanoate (VC-IP)

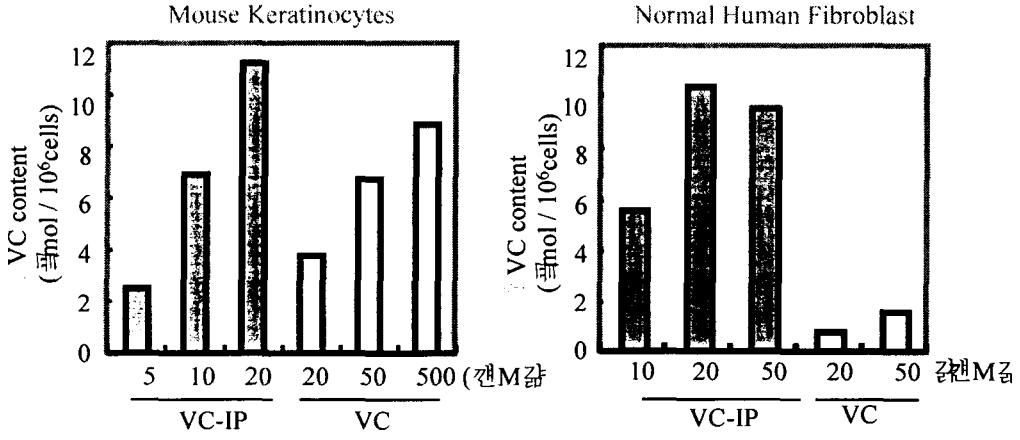

Fig.2 VC-IP could converted to VC in the cells

Cells were treated with the medium containing various concentrations of VC-IP or VC. After 2 h incubation, cells were homogenated and the content of free VC were determined using HPLC.

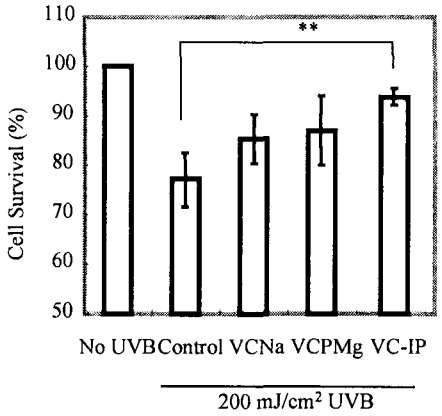

Fig.3 VC-IP reduced of the cell damages induced by UVB

HaCaT keratinocytes were treated with various 100 μM of various Vitamin C derivatives for 24 h. After 24 h from UVB irradiation, cell survival was estimated. Significance: * $p<0.05$, ** $p<0.01$.

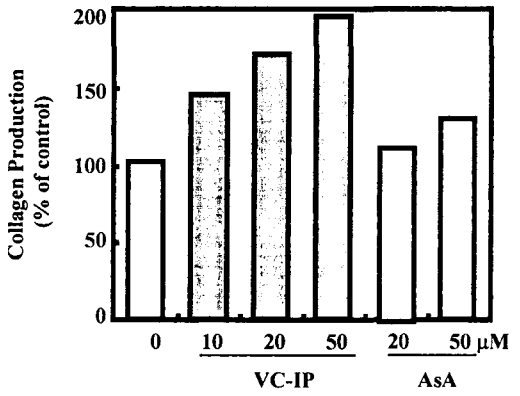

Fig.5 VC-IP increased synthesis of type I collagen

Human fibroblasts were cultured in the medium supplemented with L-[2,^{3}H]proline containing various concentration of VC-IP or ascorbic acid (AsA). After 24 hrs, cells were homogenated, uptake of L-[2,^{3}H]proline into collagenous protein was determined with syntilation counter.

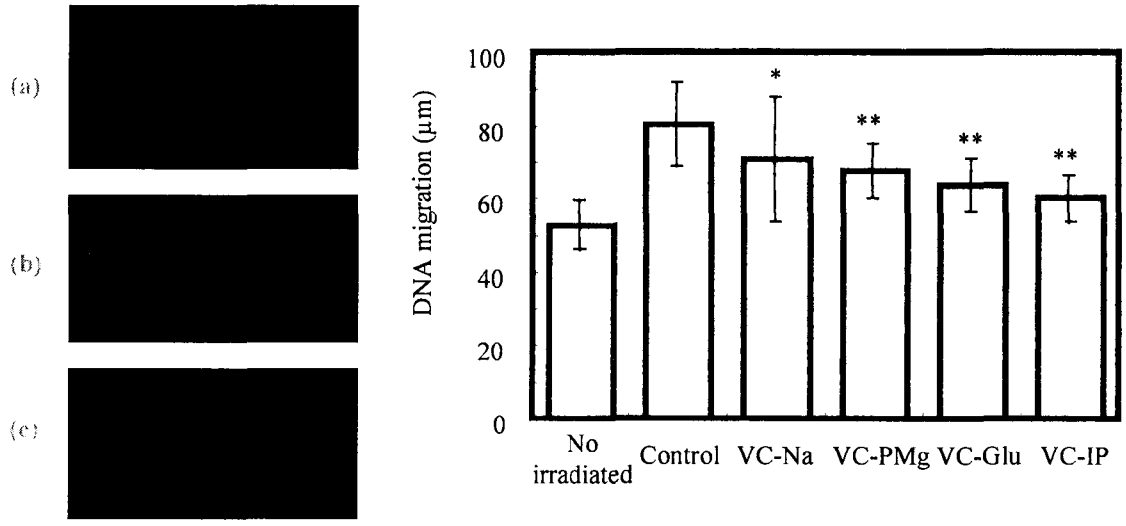

Fig.4 The ethidium bromide-stained nuclei of HaCaT cell prepared for COMET assay.

(a) Non-irradiated HaCaT cell

(b)10 mJ/cm^2 UVB irradiated HaCaT cell

(c) 10m J/cm^2 UVB irradiated HaCaT cell with 100 μM VC-IP

Significance: * $p<0.05$, ** $p<0.01$.