A New Vitamin C Based Compound for Whitening and Anti-Aging

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INTRODUCTION
In cultured melanocytes, or in mouse melanoma cells, UV-irradiation induces melanin synthesis, thus demonstrating that UV acts directly on melanocytes to induce melanogenesis (1). In addition, UV has been found to increase tyrosinase activity (2,3) and expression (4,5).

It is well known that a decrease in collagen is shown with photoaging of human skin (6,7). It has been reported that a loss of collagen may arise from an acceleration of enzymatic degradation due to collagenase release from UV-induced infiltrating cells, in which case the rate of collagen degradation exceeds the rate of biosynthesis (8). It is also well documented that UVA-irradiation stimulates the production of interstitial collagenase mRNA in cultured human skin fibroblasts (9).

The above-mentioned items can cause pigmentation and wrinkles, which is characteristic of aged skin. It is important to protect skin from these degenerative effects in daily life.

UV irradiation induces the formation of cyclobutane pyrimidine dimers and (6-4) photoproducts which may lead to cell death (apoptosis) (10). It has been reported that low-dose UV-irradiation preferentially triggers DNA repair while high-dose UV-irradiation induces apoptosis (11). Increasing apoptosis in skin leads to a decrease in the cellular proliferation and may promote skin aging. The tumor suppressor gene p53 plays an essential role in the maintenance of cellular genetic stability after a DNA-damaging event such as UV-irradiation. Following UV-irradiation, the amount of p53 protein is elevated. Thus p53 can be a candidate for the marker of DNA damage induced by UV-irradiation.

L-Ascorbic acid (AsA) plays important roles in a range of cosmetics and cosmetology, as a scavenger against reactive oxygen species and a whitening agent to inhibit melanin formation. However, AsA is not so stable when used with oxygen, acids or
alkalines, etc. in cosmetic formulations. This instability of AsA has led to the
development of derivatives of AsA with enhanced stability.

A new lipophilic AsA derivative, "2,3,5,6-O-tetra-2-hexyldecanoyl-L-ascorbic acid
(VC-IP, designated as vitamin C tetra-isopalmitate)” is especially useful because it is
chemically stabilized and exceeds AsA in terms of the above-mentioned vitamin C
potency.

In this study, we evaluated the dermatological efficacy of VC-IP as a whitening agent
and as an anti-aging agent using the following experiments: (A) the inhibitory effect on
melanogenesis in cultured human melanoma cells, (B) the promotive effect on
collagen synthesis in cultured human skin fibroblasts, (C) the inhibitory effect on matrix
metalloprotease (MMP) activity in human skin fibroblasts, (D) the inhibitory effect on
DNA damage induced by UVB (p53 expression analysis), (E) VC-IP transmembrane
uptake into the intracellular space and subsequent conversion to intracellular AsA via
enzymatic esterolysis in cultured human skin fibroblasts.

MATERIALS AND METHODS

Whitening analysis
Human melanoma cells (HM-3-KO) were incubated with VC-IP at the range of
concentration from 0% to 0.2% for 4 days. Cells were then collected by trypsinization
and centrifugation. Following cell count, slot blot was performed and the melanin
density was measured with a Pharmacia laser densitometer.

Measurement of collagen synthesis
Human skin fibroblasts NHDF cells were administered with L-[2, 3H] proline in the
presence or absence of VC-IP or AsA of 20 to 50μM for 24 h, and underwent cytolysis
by NaOH treatment and subsequent neutralization. The cell extract obtained was
thoroughly hydrolysed with collagenase from Clostridium perfringens, and separated
by TCA/tannin treatment and centrifugation to generate supernatant and precipitate as
the collagen fraction and the non-collagen protein fraction, respectively, both of which
were evaluated with an Aloka liquid scintillation counter LC-3600 sci.

Measurement of MMPs
Serum-free conditioned media of NHDF cells cultured for 48 h in the presence or
absence of 50μM VC-IP were concentrated by ultrafiltration, and were electrophoresed
under non-reduced conditions on an SDS-polyacrylamide gel containing 0.2% gelatin, followed by staining with Coomasie Brilliant Blue R250 and subsequent measurement by laser densitometry.

**Western blot analysis**
Human fibroblasts NB1RGB were incubated in a 96-well plate with media containing VC-IP at the range of concentrations from 0% to 0.01% for 24 h. The media were replaced by phosphate-buffered saline (PBS) and UVB-irradiation at 0.1 J/cm² was performed. Following UV-irradiation, cells were further incubated in media containing VC-IP at each concentration for 24 h.

The media were discarded and the cell layer was rinsed with PBS. The cells were homogenized, underwent extraction with Laemmli buffer and boiled for each sample. After electrophoresis, electrottransfer to nitrocellulose was carried out for 1 h for immunoblotting.

The filter was incubated with anti-p53 monoclonal antibody (Ab-6) against both wild and mutant type of human p53 protein for 1 h at room temperature. After washing, blots were incubated with HPR-conjugated anti-mouse IgG antibody for 1 h at room temperature. The blot was washed and detected by densitometry.

**Measurement of intracellular AsA**
NHDF were administered with VC-IP or AsA of 20 to 50µM for 2 h, and were subjected to homogenization and extraction in the presence of 3% metaphosphoric acid and 0.2% ethanol, followed by HPLC separation on an ODS column and UV/coulometric ECD detection with 0.1M KH₂PO₄-H₃PO₄ buffer (pH 2.35) containing 0.02% ethanol and 0.1mM EDTA-2Na as the mobile phase.

**RESULTS AND DISCUSSION**

*The inhibitory effect on melanogenesis in human melanoma cells*
VC-IP showed a decrease in the melanin synthesis from 72 to 12% compared to the control. This effect could be caused by the ability of VC-IP to inhibit tyrosinase activation (data not shown). Thus VC-IP may contribute to protection of skin from melanogenesis induced by UV.

[Figure 1]
The promotive effect on collagen synthesis in human skin fibroblasts
VC-IP increased the collagen synthesis to 170-195% in human skin fibroblast at the range of VC-IP concentration from 20 to 50 μM, while AsA increased it to 112-127% at the same concentration range. The enhanced efficacy of VC-IP was approximately 3.5-fold higher than AsA at each concentration. Thus VC-IP could prevent skin from forming wrinkles and has the potential to be an anti-aging agent.
[Figure 2]

The inhibitory effect on MMPs activity in human skin fibroblasts
The activity of MMP-2 and MMP-9, which degrade gelatin, were suppressed to levels as low as 18-23% and 10-17%, respectively, for administration with VC-IP (20-50 μM). Both MMPs activities were 39-46% and 43-52%, respectively, for administration with AsA (20 - 50μM). Thus VC-IP can inhibit the activity of MMPs and can be expected to suppress the increasing of collagenase induced by UV-irradiation.
[Figure 3]

The inhibitory effect on DNA damage induced by UVB (p53 expression analysis)
The expression of p53 was suppressed to 68% and 58% in the administration of 0.005% and 0.01% VC-IP. p53 expression was induced by UVB at 0.1J/cm². A small amount of p53 was also found before UV-irradiation. This level of UVB intensity had no effect on the cell viability (data not shown). It was considered that UV-irradiation induced DNA damage and p53 expression was induced by DNA damage. VC-IP inhibited the increasing of p53 expression and this suggests that VC-IP could protect DNA from UV-induced damage.
[Figure 4]

Measurement of VC-IP intake and conversion to intracellular AsA in human skin fibroblasts
The intake concentration of VC-IP or AsA was 0.7 nmol/10⁶cells for VC-IP and 10.2 nmol/10⁶cells for AsA at each concentration of 20μM. At the same concentration of administered VC-IP or AsA, VC-IP achieved the accumulation of intracellular AsA approximately 14-fold more markedly than AsA itself did. Thus VC-IP can be abundantly accumulated as AsA after esterolysis in skin cells and could also exert a cytoprotective action more efficiently.
As described in Figs. 1, 2, 3 and 4, it is considered that inhibition of melanogenesis, enhancement of collagen synthesis, inhibition of MMPs activity and inhibition the increasing of p53 expression are closely related to the enrichment of the intracellular AsA in the cells.

[Figure 5]

CONCLUSION

VC-IP has several excellent advantages that can be applied in modern skin care formulation as follows:

1) It has an inhibitory effect on melanogenesis in cultured human melanoma cells and can be expected the application for whitening agents.

2) It has a promotive effect on collagen synthesis and an inhibitory effect on MMPs activity. It can be expected to be appropriate for anti-aging agents.

3) In addition, it has an inhibitory effect on the increase in p53 induced by UV-irradiation and can be expected to have a potential for protecting skin cells from UV-induced DNA damage.

4) It underwent the intake into skin fibroblasts due to its appropriate molecular hydrophobicity and gradually esterolyzed which we hypothesize resulting in the enrichment of the intercellular AsA.

In summary, we can conclude that VC-IP has several effects on whitening and anti-aging, which are important when we focus on formulating cosmetics.

REFERENCE


Aberdam E, Romero C, Ortonne J-P. Repeated UVB irradiations do not have the same potential to promote stimulation of melanogenesis in cultured normal human


Figure 1  The inhibitory effect of VC-IP on melanogenesis in cultured human melanoma cells

Figure 2  The enhancement effect of VC-IP or AsA on collagen synthesis in human
Figure 3  The inhibitory effect of VC-IP or AsA on MMP-2 and MMP-9 activity

Figure 4  The inhibitory effect of VC-IP on DNA damage induced by UVB (p53
Figure 5  The intracellular intake of AsA or VC-IP and subsequent conversion to AsA in human skin fibroblasts