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A new lipophilic pro-vitamin C, tetra-isopalmitoyl ascorbic acid (VC-IP), prevents UV-induced skin pigmentation through its anti-oxidative properties

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Vitamin C;
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Summary

Background: Vitamin C, which is a strong anti-oxidant, plays an important role in maintaining physiological states. In dermatology, Vitamin C is used for treatment of various skin problems such as de-pigmentation of hyperpigmented spots. However, Vitamin C has limited stability and permeability, and development of a Vitamin C derivative with improved properties is needed.
Objective: We evaluated the effect of a lipophilic Vitamin C derivative, tetra-isopalmitoyl ascorbic acid (VC-IP), on ultraviolet (UV)-induced skin pigmentation, to determine its potential as a more effective form of Vitamin C.
Methods: The release of Vitamin C from VC-IP was examined using a reconstructed skin model following topical application of VC-IP. Anti-oxidative and anti-inflammatory activities of VC-IP were tested in cultured human keratinocytes. Subsequently, clinical test was done to clarify the effect of VC-IP on UVB-induced skin pigmentation.
Results: VC-IP released Vitamin C in physiological conditions and worked as pro-Vitamin C. In subsequent experiments, we found that VC-IP suppressed the elevation of intracellular peroxide after UVB irradiation, and enhanced cellular tolerance against UVB and reactive oxygen species such as hydrogen peroxide and *tert*-butyl hydroperoxide. Furthermore, VC-IP reduced the production of interleukin-1α and prostaglandin E2 in UVB-irradiated keratinocytes and suppressed melanocyte prolif-

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eration in conditioned culture medium prepared from UVB-irradiated keratinocytes. Finally, in a clinical study, topical application of a 3% VC-IP cream for 3 weeks suppressed pigmentation after UVB irradiation.

Conclusions: These results demonstrate that VC-IP is a precursor of Vitamin C, and effectively suppresses UVB-induced skin pigmentation, possibly through its anti-oxidative activity.

1. Introduction

Vitamin C, one essential substance needed to maintain the normal physiological state in humans, is well known as a multifunctional vitamin. In the skin, Vitamin C is an essential substance in the bio-synthesis of collagen, which plays an important role in the structure of the dermis, because Vitamin C is a cofactor required for the enzymatic activity of prolyl hydroxylase, which hydroxylates prolyl residues in procollagen and elastin [1—3]. Recent studies have reported new functions for Vitamin C as contributing to the formation of skin barrier function by enhancing epidermal differentiation [4,5] and by stimulating blood flow through nitric oxide (NO) production via increases in the stability of tetrahydrobiopterin, a cofactor of constitutive NO synthase [6].

In general, Vitamin C is a natural anti-oxidant and there are many reports on its anti-oxidative capability. Exposure of the skin to ultraviolet (UV) generates reactive oxygen species (ROS) [7] and causes adverse effects by oxidative stress such as DNA damage [8] and suppression of the skin immune system [9]. Topical application of Vitamin C improves these adverse effects [10—15]. In addition, Vitamin C has been widely used topically and orally for treating skin disorders such as hyperpigmented spots [16,17]. However, in the case of topical application, fresh Vitamin C must be applied to the skin by iontophoresis to achieve clinical improvement, because of its poor penetration of the skin and its instability in formulations [18,19]. To cover these defects of Vitamin C, many kinds of derivatives, such as magnesium L-ascorbyl-2-phosphate (VC-PMg) [20], ascorbic acid 2-*O*-alpha-glucoside [21], ascorbyl palmitate [22] and 6-acylated ascorbic acid 2-*O*-alpha-glucoside [23] have been synthesized.

In this study, we characterized the capabilities of a newly synthesized lipophilic Vitamin C derivative, tetra-isopalmitoyl ascorbic acid (VC-IP), as a pro-Vitamin C (Fig. 1) and we evaluated the effect of VC-IP on UVB-induced skin pigmentation. VC-IP has no inherent capabilities as an anti-oxidant, because all hydroxy groups in Vitamin C are esterified with isopalmytic acid. Thus, it is essential that Vitamin C be released from VC-IP in human tissues or cells to obtain any anti-oxidative effects. We found that VC-IP is a precursor of Vitamin C, and reduces cell damage triggered by UVB and by ROS. In addition, VC-IP effectively suppresses UVB-induced pigmentation. We discuss the possible mechanism by which VC-IP suppresses UV-induced melanogenesis from the point of view of its anti-oxidative properties.

2. Materials and methods

2.1. Reagents

VC-IP and VC-PMg were synthesized by Nihon Surfactant Co. Ltd. (Tokyo, Japan). 3-(4,5-Dimethy-l-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dihydrorhodamine123 (DHR123), hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (t-BHP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco's modified Eagle medium (DMEM) was obtained from Nikken Bio Medical Laboratory (Kyoto, Japan). Fetal bovine serum (FBS), human normal keratinocytes, human normal melanocytes and Humedia KG2 were purchased from Kurabo (Osaka, Japan).

2.2. Release of Vitamin C from VC-IP

The conversion of VC-IP to Vitamin C was assessed using a combination of a reconstructed skin model (TEST SKIN LSE-high LSE003 (Toyobo, Tokyo, Japan)) and high performance liquid chromatography (HPLC) analysis. Vehicles with or without VC-IP were applied to the top of the reconstructed skin loaded in Franze type diffusion cells. As a receiver solution, 40% PEG400 in phosphate buffered saline (PBS) was

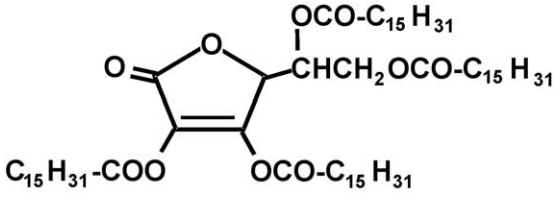

Fig. 1 Chemical structure of VC-IP.

used. The reconstructed skin was incubated at 37 °C for 48 h, after which it was immersed in 2 ml of extracting solution (containing 0.9% perchloric acid and 0.1% dithiothreitol (DTT)) and was sonicated on ice for 15 min. One ml of the extract, which was clarified by passage through a 0.45 μm membrane filter (SUN Sri), was used for HPLC analysis to detect Vitamin C. HPLC conditions were as follows: column, ULTRON PS-80H (300 mm × 8 mm Ø, Shinwakakou, Co. Ltd., Tokyo, Japan); mobile phase, 0.18% perchloric acid; detection, 254 nm. The HPLC system was composed of a pump, LC-10ADVP and detector, SPD-10AVP (Shimazu Seisakusyo, Kyoto, Japan).

2.3. UVB irradiation

The UVB source was Toshiba fluorescent sunlamps (FL-20SE.30, Tokyo, Japan) which an emission spectrum from 270 to 375 nm, peaking at 305 nm. The irradiance of UVB was measured by a UV light meter UV-340 (Lutron, Coopersburg, PA, USA).

2.4. Tolerance against oxidative stress

HaCaT keratinocytes were placed in 96-well plates at a density of 3.5×10^4 cells/well. After 1 day of cultivation, cells were pre-cultured in DMEM containing 100 μM VC-IP and 5% FBS for 24 h. They were then exposed to sources of oxidative stress as follows: (1) UVB, 200 mJ/cm^2 in Hanks' balanced salt solution (HBSS); (2) H_2O_2, 5 mM in HBSS for 2 h; (3) t-BHP, 0.3 mM for 2 h. Cell viability was estimated by the neutral red assay immediately after oxidative stress treatment. In the case of UVB irradiation, cell viability was measured 24 h following UVB irradiation. Survival ratios are expressed as percentages of UVB non-irradiated cells

2.5. Intracellular peroxide level [24]

HaCaT keratinocytes were placed in 96-well plates at a density of 3.5×10^4 cells/well. After 1 day of cultivation, cells were treated with DMEM containing various concentrations of VC-IP and 5% FBS for 24 h, and were then irradiated with 100 mJ/cm^2 UVB in HBSS. Following UVB irradiation, cells were incubated in DMEM containing 5% FBS and 20 μM DHR123 at 37 °C for 45 min. The cells were lysed and the fluorescence (Ex, 480 nm; Em, 539 nm) was measured using a fluorescence micro-plate reader (Spectra Max Gemini, Molecular Devices, Sannwel, CA, USA). Peroxide levels are expressed as fluorescence intensity (F.I.) per microgram protein. Protein concentrations were determined using the bicinchroninic acid (BCA)-protein assay kit (Pierce, Rockfold, IL, USA).

2.6. Melanocyte proliferation in UVB-irradiated keratinocyte conditioned medium

Normal human keratinocytes were inoculated at a density of 30×10^4 cells in 35 mm Ø dishes. After 1 day of cultivation, cells were pre-cultured in Humedia KG2 containing various concentrations of VC-IP for 24 h. Cells were irradiated with 5 mJ/cm^2 UVB in HBSS. Following UVB irradiation, cells were cultured in Humedia KG2 containing appropriate concentrations of VC-IP for 24 h.

Normal human melanocytes were placed in 96-well plates at a density of 3.5×10^4 cells/well. After 1 day of cultivation, cells were incubated in the UVB-irradiated keratinocyte conditioned medium for 24 h. The proliferation of melanocytes was estimated using the MTT assay.

2.7. IL-1α and PGE_2 secretion following UVB irradiation

Normal human keratinocytes were placed in 96-well plates at a density of 3.5×10^4 cells/well. After 1 day of cultivation, cells were pre-cultured in Humedia KG2 containing various concentrations of VC-IP for 24 h. Cells were then irradiated in HBSS at a UVB dose of 5 mJ/cm^2. After UVB irradiation, the cells were cultured in fresh Humedia KG2 for 24 h. Interleukin-1α (IL-1α) and prostaglandin E2 (PGE_2) secreted into the culture medium were quantified using commercial assay enzyme-linked immunosorbent Assay (ELISA) kits, Human IL-1α Immunoassay (Quantikine, Minneapolis, MN, USA) and PGE_2 EIA (Cayman, Ann Arbor, MI, USA).

2.8. Human clinical study

This study was performed using randomized application sites in a double-blind paired design during February—March 2005, in Osaka, Japan. Twenty-two Japanese males and females, aged 20—40 years, with characteristic Japanese photo-skin type II or III were enrolled. In the study, the effect of VC-IP on UV-induced skin pigmentation was evaluated. The inner side of the upper arm was used for testing as the site of sun-protected skin. Subjects were exposed to 1.5 minimal erythema dose (MED) of solar-simulated light with a Multiport Solar UV Simulator Model 601 (Solar Light Co., Inc., Glenside, PA, USA). Following exposure, a cream containing 3% VC-IP or vehicle only (oil in water (O/W) type cream) was topically applied to the UV-irradiated area immediately after irradiation. After 1, 2 and 3 weeks, intensities of pigmentation were evaluated with L^*-value measurement and were scored visually

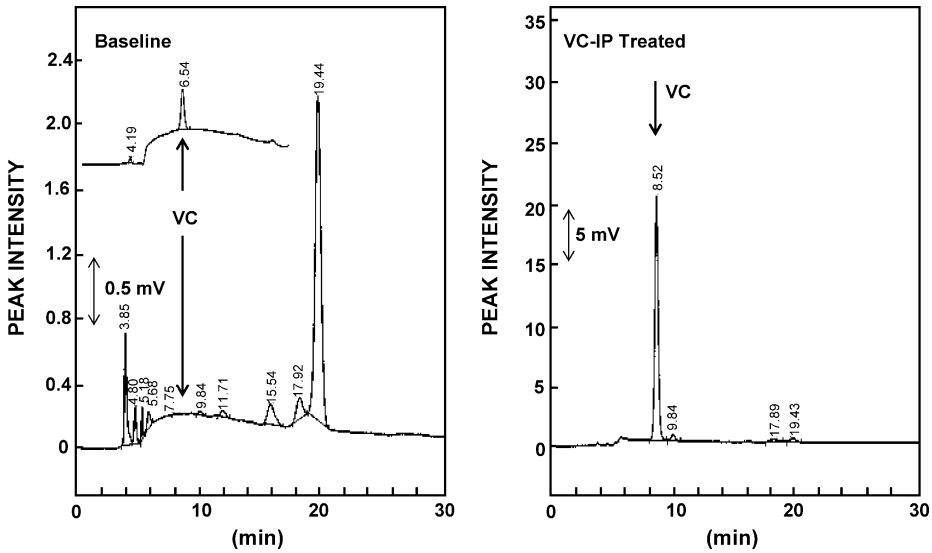

Fig. 2 Conversion of VC-IP to Vitamin C, the conversion of VC-IP to Vitamin C was assessed using a combination of a reconstructed skin model and HPLC analysis.

by an expert judge. L^*-values were measured with a Minolta Chromameter (Model CR-13, Osaka, Japan). Changes in L^*-value are expressed as ΔL^* (=L^* of each week − L^* before UV-irradiation). Pigmentation was visually rated on a score of 1—4: 1, no difference, 2, small difference, 3, moderate difference or 4, large difference, compared to the color of a neighboring normal skin area. Differences in ΔL^*-values and visual scores were analyzed using the Wilcoxon signed rank test. All subjects gave written informed consent, and the protocol had been approved by the regulatory and safety review committee for human testing of the Cosmos Technical Center Co. Ltd.

3. Results

3.1. Release of Vitamin C from VC-IP

To characterize the potential of VC-IP as a pro-Vitamin C, we measured levels of Vitamin C in the VC-IP treated reconstructed skin. By HPLC analysis, 694.8 ± 197.5 nmol/cm^2 Vitamin C was quantified in homogenates of VC-IP treated skin, while no peak could be detected in the untreated control skin (Fig. 2). At the same time, 133.3 ± 28.1 nmol/cm^2 VC-IP was quantified in the VC-IP treated skin. These results indicate that VC-IP is converted to Vitamin C at a ratio of 84% in the reconstructed skin model.

3.2. Attenuation of oxidative stress

We tested the effect of VC-IP to suppress oxidative stress, including UVB and ROS such as hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (t-BHP). Toxicity test indicated that treatment with 200 μM of VC-IP exhibited the decrease of cell survival. Thus, we decided 100 μM of VC-IP was the maximum concentration of the following experiments. The effect of VC-IP on the survival of HaCaT keratinocytes exposed to UVB-irradiation or ROS are summarized in Table 1. VC-IP enhanced the survival of HaCaT cells against all 3 types of exogenous oxidative stress tested. We then assessed the effects of VC-IP on the level of intracellular peroxide after UVB irradiation. UVB irradiation significantly elevated levels of intracellular peroxide by 120% (Fig. 3). Pre-treatment with VC-IP before UVB irradiation abrogated the elevation of intracellular peroxide in a dose-dependent manner.

Table 1 Protective effect of VC-IP against cell damage induced by oxidative stress, UVB, H_2O_2 and t-BHP

	μM	Tolerance against UVB		Tolerance against H_2O_2		Tolerance against t-BHP	
		Survival (%)	*t*-Test	Survival (%)	*t*-Test	Survival (%)	*t*-Test
Control	0	77.07 ± 5.52		38.16 ± 1.46		74.58 ± 5.90	
VC-IP	100	93.83 ± 1.72	**	45.59 ± 3.52	**	103.31 ± 1.60	**

Data are expressed as average ± standard deviation (n = 4).
** Significance (Student's *t*-test): $p < 0.01$.

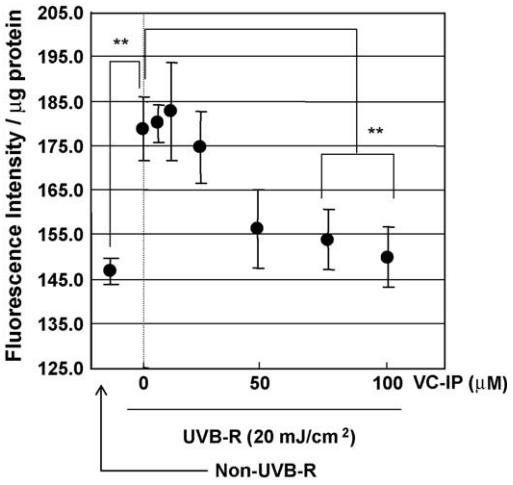

Fig. 3 Intracellular peroxide levels in HaCaT keratinocytes after UVB irradiation, HaCaT keratinocytes were exposed to UVB at 100 mJ/cm^2 after treatment with VC-IP for 24 h. Following UVB irradiation, cells were incubated in 20 μM DHR123 at 37 °C for 45 min. After lysis of the cells, the fluorescence (Ex, 480 nm; Em, 539 nm) was measured. Peroxide levels are expressed as fluorescence intensity (F.I.) per μg protein. $n = 4$. Significance (Student's t-test): $^{**}p < 0.01$.

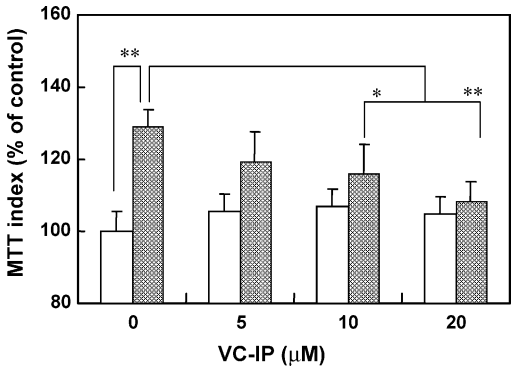

Fig. 4 Melanocyte proliferation in conditioned medium prepared from UVB-irradiated keratinocytes, normal human keratinocytes were exposed to UVB at 5 mJ/cm^2 after treatment with VC-IP for 24 h. Conditioned media were prepared by cultivating UVB irradiated-cells in fresh Humedia KG2 containing VC-IP for 24 h. Normal human melanocytes were cultured with each conditioned medium for 24 h. The proliferation of melanocytes was measured by MTT assay. (□) Sham UVB-irradiated, (▨) UVB-irradiated. $n = 6$. Significance (Student's t-test): $^{*}p < 0.05$, $^{**}p < 0.01$.

These results indicate that VC-IP reduces damage by oxidative stress and suppresses the production of intracellular peroxide generated by oxidative stress.

3.3. Suppression of melanocyte proliferation factors derived from keratinocytes

To further characterize the potential of VC-IP to suppress UVB-induced skin pigmentation, we assessed the effects of VC-IP on the secretion of melanocyte proliferation factors from UVB-irradiated keratinocytes. We prepared conditioned medium from UVB-irradiated keratinocytes treated or not treated with VC-IP. The conditioned medium obtained from UVB-irradiated keratinocytes without VC-IP pretreatment increased melanocyte proliferation by 130% (Fig. 4). In contrast, the conditioned medium prepared from UVB treated keratinocytes pretreated with VC-IP suppressed melanocyte proliferation in a dose-dependent manner. To understand the mechanism underlying the effect of VC-IP on melanocyte proliferation, we examined the effects of VC-IP on the release of IL-1α and PGE_2, which are important factors that stimulate melanocyte proliferation. VC-IP abrogated the elevated secretion of IL-1α and PGE_2 following UVB irradiation of keratinocytes (Figs. 5 and 6). These results indicate that VC-IP suppresses secretion of the melanocyte proliferation factors, IL-1α and PGE_2, from UVB-irradiated keratinocytes. Further, they suggest that VC-IP may have a suppressive effect on UVB-induced skin pigmentation.

3.4. Human clinical study: preventive effects of VC-IP on UVB-induced skin pigmentation

Tables 2 and 3 show the visual scores and ΔL^*-values of UVB-irradiated areas treated with vehicle cream

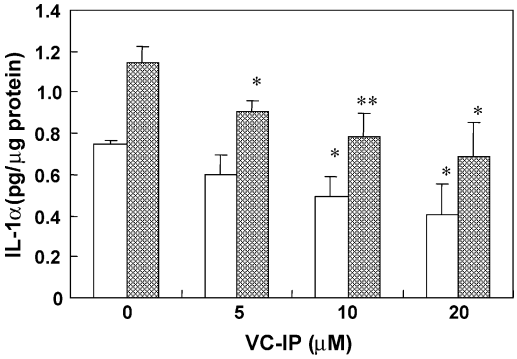

Fig. 5 IL-1α production by human normal keratinocytes following UVB irradiation, normal human keratinocytes were exposed to UVB at 5 mJ/cm^2 after treatment with VC-IP for 24 h. After UVB irradiation, the cells were cultured in fresh Humedia KG2 for 24 h. IL-1α levels in the cultured media were quantified using an Human IL-1α Immunoassay (□) sham UVB-irradiated, (▨) UVB-irradiated. $n = 6$. Significance (Student's t-test) $^{*}p < 0.05$, $^{**}p < 0.01$.

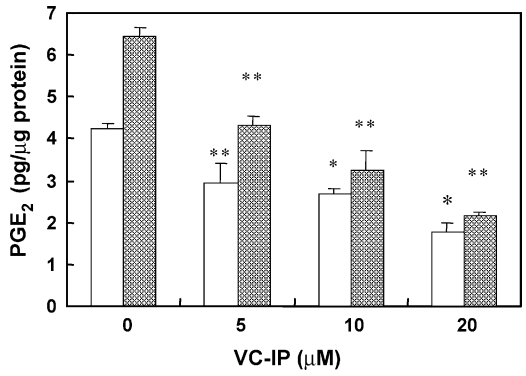

Fig. 6 PGE_2 production by human normal keratinocytes following UVB irradiation, Normal human keratinocytes were exposed to UVB at 5 mJ/cm^2 after treatment with VC-IP for 24 h. After UVB irradiation, the cells were cultured in fresh Humedia KG2 for 24 h. PGE_2 levels in the cultured media were quantified using a PGE_2 EIA. (□) Sham UVB-irradiated, (▨) UVB-irradiated. $n = 6$. Significance (Student's t-test): $^{*}p < 0.05$, $^{**}p < 0.01$.

or with 3% VC-IP cream at various times after UVB exposure. We obtained statistically significant differences between vehicle treated areas and VC-IP treated areas 1 week after UVB irradiation ($p < 0.05$) by visual scoring. ΔL^{*}-values of VC-IP treated areas gave significantly lower values than those of vehicle treated areas at 1 week and at 2 weeks after UVB irradiation ($p < 0.05$). These observations indicate that VC-IP suppresses UVB-induced skin pigmentation.

4. Discussion

We initially examined whether the synthetic derivative VC-IP is converted to Vitamin C physiologically. In general, the active sites of Vitamin C are the hydroxy groups at the 2 and 3 positions. Since VC-IP is blocked by isopalmitoyl residues at all hydroxyl groups, VC-IP itself is inactive in scavenging against radicals or peroxides. To exert anti-oxidative effects, VC-IP would be required to be converted to Vitamin C in cells through the enzymatic reaction of cytosolic esterase. HPLC analysis showed the efficient release of Vitamin C in reconstructed skin treated with VC-IP, demonstrating that VC-IP acts as pro-Vitamin C (Fig. 2). In addition, VC-IP were examined multiple safety tests, e.g. human sensitization test, closed patch test, eye irritation test, mutation test, and all tests were negative, which demonstrates that there are few possibilities of tissue damage or carcinogenicity for VC-IP itself.

In this study, we clarified the capabilities of VC-IP to act as pro-Vitamin C and showed the suppressive effect of VC-IP on UVB-induced skin pigmentation (Tables 2 and 3). The possible mechanisms of VC-IP to suppress UVB-induced skin pigmentation can be discussed, focusing on its anti-oxidative capabilities.

The mechanisms of UVB induced-skin pigmentation have been well studied with respect to the roles of cytokines (IL-1, endothelin-1 (ET-1), stem cell factor (SCF)), hormones (adrenocorticotrophin, α-melanocyte stimulating hormone (α-MSH)) and inflammatory chemical mediators (PGE_2) [25–29].

Table 2 Human study: visual score, number of subjects with each score at various times after UVB irradiation

Score	1 week		2 weeks		3 weeks	
	Vehicle	VC-IP	Vehicle	VC-IP	Vehicle	VC-IP
1	0	1	0	0	1	0
2	6	11	9	11	8	13
3	3	2	1	1	0	0
4	13	8	12	10	13	9
Significance	*		ns		ns	

ns, not significant.
* Wilcoxon signed rank test, $p < 0.05$.

Table 3 Human study, ΔL^{*}-values of VC-IP treated sites at various times after UVB irradiation

	1 week		2 weeks		3 weeks	
	Vehicle	VC-IP	Vehicle	VC-IP	Vehicle	VC-IP
Average	3.36	2.93	2.76	2.31	2.03	1.70
S.D.	2.05	1.85	2.21	2.15	1.75	1.65
Significance	*		*		ns	

S.D.: standard deviation; ns, not significant.
* Wilcoxon signed rank test, $p < 0.05$.

It has been reported that the relevant cytokines, hormones and mediators (IL-1α, α-MSH, PGE_2) which regulate skin pigmentation are stimulated through the ROS pathway [30—32]. In addition, ROS can contribute to skin pigmentation directly. ROS, superoxide anion radicals, H_2O_2 and hydroxyl radicals, which are generated in the skin during/after UVB-irradiation, due in part to activation of the mitochondrial respiratory chain reaction [7] and nicotineamide adenine dinucleotide phosphate (NADPH) oxidase [33], accelerate melanin formation by regulating tyrosinase enzymatic activity [34].

In this study, we focused on IL-1α and PGE_2 which are produced by UVB-irradiated keratinocytes. IL-1α is a trigger for melanocyte proliferation and tyrosinase gene expression through ET-1 relevant signal transduction [25]. PGE_2 also works to stimulate skin pigmentation by melanocyte activation involving cell growth, up-regulation of tyrosinase mRNA and development of dendrites [28,29]. Also, it is well established that IL-1α and PGE_2 are regulated by ROS [32] and that IL-1α increases oxidative stress [35]. Therefore, IL-1α and PGE_2 seem to be suitable for estimating the potential of VC-IP to suppress UVB-induced skin pigmentation from the aspect of oxidative stress. Other cytokines, hormone and growth factors are also known to activate melanocytes. Since some of them, such as α-MSH, are also up-regulated by ROS [31], it is possible that VC-IP also regulate their action.

The possibility that VC-IP suppresses UVB-induced pigmentation via ROS scavenging and reducing secretion of melanocyte proliferation factors was examined. We initially examined the effects of VC-IP on tolerance against ROS-induced cell damage. The protective effect of VC-IP on cells exposed to various types of oxidative stress such as UVB, H_2O_2 and t-BHP was examined. VC-IP treated HaCaT keratinocytes showed significantly higher resistance against oxidative stress-induced cell death compared with non-treated cells (Table 1). In addition, treatment with VC-IP significantly reduced peroxide levels in cells with or without UVB irradiation (Fig. 3). These results indicate that VC-IP effectively reduces oxidative stress, possibly due to the release of Vitamin C.

To clarify the effects of VC-IP on melanocyte proliferation, we examined the effects of conditioned medium from UVB-irradiated keratinocytes and quantified IL-1α and PGE_2 levels in those conditioned media. Melanocyte proliferation in conditioned medium prepared from keratinocytes treated with VC-IP was suppressed compared with conditioned medium prepared from non-treated keratinocytes (Fig. 4). In addition, the corresponding result was also exhibited using by conditioned medium from keratinocytes with only pretreatment of VC-IP (data not shown).

Further we found significant decreases in IL-1α and PGE_2 in media from keratinocytes treated with VC-IP (Figs. 5 and 6). These results indicate that VC-IP reduces the UVB-induced elevation of melanin production, possibly through the suppression of IL-1α and PGE_2 secretion by keratinocytes. Correspondently, clinical test for VC-IP was evaluated in Japanese volunteers and exhibited significant skin-lightening effects after 1 week application. However, after 2 and 3 weeks application, the difference between 3% VC-IP cream and vehicle cream became smaller (Tables 2 and 3). That reason is presumed that the suntanned skin color became the basal level again. Thus, VC-IP would contribute to the initial step of suntan formation, which is consistent in the other results (Figs. 5 and 6). In addition, there is a possibility of race differences or skin type differences for VC-IP action, because inflammatory responses against UVB irradiation depend on individual skin types.

Taken together, these results demonstrate that VC-IP is effectively converted to Vitamin C in vitro and that it reduces oxidative stress induced by environmental stimuli. In addition, we found that VC-IP reduces the production of UVB-induced melanocyte proliferation factors, IL-1α and PGE_2, by keratinocytes. We conclude that VC-IP suppresses UVB-induced skin pigmentation by the ROS scavenging pathway.

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