Inhibitory effect of magnesium L-ascorbyl-2-phosphate (VC-PMG) on melanogenesis in vitro and in vivo

Koichiro Kameyama, MD, PhD,[a] Chie Sakai, MD,[a] Shigeo Kondoh, MD,[a] KohZoh Yonemoto, MD, PhD,[a] Shigeo Nishiyama, MD, PhD,[a] Masato Tagawa,[b] Tomoji Murata,[b] Toshio Ohnuma,[b] John Quigley,[c] Albert Dorsky,[c] Daniel Bucks,[c] and Kurt Blanock[c] *Sagamihara and Tokyo, Japan, and Foster City, California*

Background: An inhibitory effect of ascorbic acid (AsA) on melanogenesis has been described. However, AsA is quickly oxidized and decomposed in aqueous solution and thus is not generally useful as a depigmenting agent.
Objective: Our purpose was to examine the effect on pigmentation of magnesium-L-ascorbyl-2-phosphate (VC-PMG), a stable derivative of AsA.
Methods: Percutaneous absorption of VC-PMG was examined in dermatomed human skin, and its effect on melanin production by mammalian tyrosinase and human melanoma cells in culture was also measured. A 10% VC-PMG cream was applied to the patients.
Results: VC-PMG suppressed melanin formation by tyrosinase and melanoma cells. In situ experiments demonstrated that VC-PMG cream was absorbed into the epidermis and that 1.6% remained 48 hours after application. The lightening effect was significant in 19 of 34 patients with chloasma or senile freckles and in 3 of 25 patients with normal skin.
Conclusion: VC-PMG is effective in reducing skin hyperpigmentation in some patients.
(J AM ACAD DERMATOL 1996;34:29-33.)

Lack of L-ascorbic acid (AsA, vitamin C) is a key factor in scurvy.[1] AsA also affects collagen synthesis,[2,3] drug-metabolizing enzyme,[4] production of interferon,[5] antiviral,[6] antibacterial,[7] and antioxidant actions.[8,9] Tyrosinase [EC1.14.18.1] is a key enzyme involved in melanin production in mammals.[10,11] Tyrosinase has three catalytic functions[12] and its structural gene has been cloned and mapped to the mouse albino locus on chromosome 7[13] and to the human TYR locus on chromosome 11.[14,15] However, many other factors such as tyrosinase-related proteins 1 (TRP1) and 2 (TRP2) are involved in the regulation of melanin synthesis in mammals.[16-19] These interact to modulate the quantity and quality of the pigment produced. In addition, an endogenous heat-resistant melanogenic inhibitor has also been described that interrupts the production of melanin, even in the presence of active tyrosinase.[20] We have previously reported that tyrosinase, TRP1, TRP2, and the melanogenic inhibitor are important in the regulation of melanin production by murine melanoma cells.[21] AsA inhibits melanin production by reducing *o*-quinones,[22] so that melanin cannot be formed by the action of tyrosinase until all AsA is oxidized. A more recent report also suggests a reducing effect of vitamin C on *o*-quinones.[23] Melanin can be changed from jet black to light tan by AsA by the reduction of oxidized melanin.[22] The disadvantage of AsA is that it is quickly oxidized and decomposes in aqueous solution. To resolve that problem, magnesium-L-ascorbyl-2-phosphate (VC-PMG) was synthesized.[24] Although, the structure of VC-PMG was first assumed to be magnesium-L-ascorbate-3-phosphate, it was suggested later that the structure of VC-PMG is magnesium-L-ascorbyl-2-phosphate.[25] VC-PMG is stable in water, especially in neutral or alkaline solution containing boric acid or its salt.[26] VC-PMG is hydrolyzed by phosphatases of liver or skin to AsA and thus exhibits vitamin C–reducing activity.[26] In this study we investigated

From the Department of Dermatology, Kitasato University School of Medicine, Sagamihara[a]; Nikko Chemicals Co., Ltd., Tokyo[b]; and Penederm, Inc., Foster City.[c]

Accepted for publication June 6, 1995.

Reprint requests: Koichiro Kameyama, 3-12-10 Yagumo, Park Villa 2F, Meguro, 152, Japan.

0190-9622/96 $5.00 + 0 **16/1/66884**

Table I. Effect of VC-PMG on melanin formation by purified tyrosinase*

Concentration of VC-PMG	Result (pmol/16 hr)	% Inhibition
Control	18 ± 2	
1.0%	3 ± 0.2	82 ± 4
1.0^{-1}%	3 ± 0.2	83 ± 1
1.0^{-2}%	2 ± 1	87 ± 4
1.0^{-3}%	2 ± 1	33 ± 5
1.0^{-4}%	16 ± 1	11 ± 8
1.0^{-5}%	16 ± 2	6 ± 11
1.0^{-6}%	18 ± 1	−2 ± 4
1.0^{-7}%	17 ± 1	3 ± 5

*Data are expressed as percent suppression of a standard aliquot of purified mammalian tyrosinase and represent the means of assays carried out in tetraplicate ± SD.

Table II. Effect of VC-PMG on melanin formation by B16F10 cell extract*

Concentration of VC-PMG	Result (pmol/10^5 cells/hr)	% Inhibition
Control	34 ± 5	
1.0%	3 ± 1	92 ± 3
1.0^{-1}%	3 ± 1	92 ± 2
1.0^{-2}%	32 ± 1	7 ± 3
1.0^{-3}%	39 ± 2	−14 ± 6
1.0^{-4}%	35 ± 1	−4 ± 4

*Data are expressed as percent suppression of a standard aliquot of B16F10 cell extract and represent the means of assays carried out in tetraplicate ± SD.

the effects of VC-PMG on melanogenesis in vitro and in vivo, as well as its percutaneous absorption.

MATERIAL AND METHODS

Cell lines and culture techniques

B16F10 murine melanoma and KHm-¼ human melanoma cells were used.[27,28] KHm-¼ cells (1×10^6) were seeded in 75 cm^2 flasks; 24 hours later, the media were changed, and VC-PMG (at 1×10^{-10} to 1%) was added where noted. The media were changed daily thereafter. After 3 days treatment, the cells were harvested, counted, and solubilized with 1% Nonidet P40 (NP40), and melanogenic activities determined, as detailed later.

Melanogenic activity assay

Melanogenic activity was measured by radiometric assays using [U-^{14}C] tyrosine for total melanin production as previously described.[29]

Table III. Effect of VC-PMG on cultured KHm-1/4 human melanoma cells*

Concentration of VC-PMG	Result (pmol/10^5 cells/hr)	% Inhibition	Cell number ($\times 10^6$)
Control	40 ± 3		1.1 ± 0.4
1.0%	21 ± 2	48 ± 5	0.9 ± 0.5
0.5%	30 ± 2	25 ± 6	1.0 ± 0.4
0.1%	36 ± 1	9 ± 3	1.1 ± 0.4
0.05%	38 ± 2	5 ± 7	1.1 ± 0.4
0.01%	41 ± 2	−2 ± 5	1.1 ± 0.3

*KHm-1/4 cells were cultured with or without VC-PMG for 3 days, harvested, and solubilized as described. The results represent the means of assays carried out in tetraplicate ± SD.

Purification of tyrosinase

Purification of tyrosinase from pigmented B16 melanoma cells was performed as previously described.[30]

Percutaneous absorption of VC-PMG into the skin

VC-PMG cream 3% was spiked with [1-^{14}C]-labeled VC-PMG. The activity of each cream formulation was determined by liquid scintillation spectrometry, and percutaneous absorption was performed as previously described.[31] At time zero, approximately 10 mg of each vehicle was pipetted onto dermatomed (530 ± 90 mm) human cadaver skin that was clamped into flow-through diffusion cells with an exposed area of 0.64 cm^2. The receptor solution consisted of phosphate-buffered saline solution at pH 7.4, containing 0.1% sodium azide. The flow rate of receptor solution was set at 1 ml/hr, and receptor samples were collected every 6 hours for 48 hours. After 48 hours, the skin surface was washed three times with swabs soaked in 1% sodium lauryl sulfate and 1% ammonium lauryl sulfate in water, then given two repetitive water swabs, and wiped dry with a single swab. Each swab was then collected for subsequent analysis of radiolabel content. The skin was then removed from the bxdiffusion cell and the epidermis was separated from the dermis with trypsin. Epidermal and dermal samples were then digested in potassium hydroxide (KOH) before analysis of radiolabel content. A 1 ml aliquot was removed from each receptor phase sample for liquid scintillation counting. The remaining receptor fluid was frozen and analyzed by high-pressure liquid chromatography for confirmation of the presence of intact VC-PMG. Radioactivity was assayed from the wipes washed on skin surface, cell top, epidermal skin, dermal skin, and receptor fluid samples. 1,1-Methyleneglycol-bis (Polyprepolymer-2) was synthesized from polypropylene glycol.

Table IV. Percutaneous absorption and penetration of VC-PMG*

Formulation	Epidermis	Dermis	Wash	Receptor	% Recovered
VC-PMG	0.70 ± 0.23	0.88 ± 0.69	86 ± 5	0.19 ± 0.12	88 ± 5
VC-PMG plus 1% 1,1-methyleneglycol-bis	0.90 ± 0.43	1.07 ± 0.57	81 ± 6	0.51 ± 0.31	84 ± 6
VC-PMG plus 3% 1,1-methyleneglycol-bis	0.63 ± 0.25	1.46 ± 1.70	88 ± 4	0.09 ± 0.02	90 ± 2
VC-PMG plus 5% 1,1-methyleneglycol-bis	0.67 ± 0.23	1.10 ± 0.67	87 ± 3	0.16 ± 0.06	89 ± 3
VC-PMG plus 7% 1,1-methyleneglycol-bis	0.58 ± 0.23	0.60 ± 0.47	93 ± 4	0.25 ± 0.13	95 ± 4

*Data are expressed as percent of applied dose. The results represent represent the means of assays carried out ± SD ($n = 5$).

Table V. Effects of VC-PMG on human skin*

Disease	No. of cases	Effective	Fairly effective	Slightly effective	Not effective	Possible darkening
Chloasma	11	1	5	4	0	1
Senile freckle	17	4	6	5	2	0
Ephelides	4	2	1	1	0	0
Nevus of Ota	2	0	0	0	1	1
Total	34	7	12	10	3	2
Healthy skin	25	1	2	8	12	2

*Data are expressed as number of cases.

Lightening effects of VC-PMG on human skin

VC-PMG cream 10% was applied twice a day to the skin of 34 patients with ephelides, chloasma, senile freckles, nevus of Ota, or healthy skin. The effectiveness of the lightening of the pigmentation was judged by a color-difference meter (Minorta CR-200b, Tokyo, Japan). If the brightness index number of color-difference meter increased more than 3.0, it was defined as effective, an increase of 2.0 to 3.0 was defined as fairly effective; an increase of 1.0 to 2.0 was defined as slightly effective; an increase less than 1.0 was defined as not effective; and a decrease of more than 1.0 was defined as possible darkening.

RESULTS

Effects of VC-PMG on melanogenesis

Purified tyrosinase showed melanin formation activity of 18 ± 2 pmol at 16 hours (Table I). VC-PMG suppressed melanin formation in a dose-dependent manner, and a concentration of at least 10^{-3}% significantly suppressed melanin formation by tyrosinase. After VC-PMG was added to the cell extract of B16, ^{14}C-tyrosine assay was similarly performed (Table II). VC-PMG 0.1% or 1% significantly suppressed melanin formation more than 90%. KHm-¼ cells showed 40 pmol melanin formation/10^5 cells per hour without VC-PMG; when the cells were cultured with 1% VC-PMG for 3 days, melanin formation was inhibited by 48% ± 5% and cell growth was slightly suppressed (Table III). When the cells were cultured with 0.5% VC-PMG, melanin formation was inhibited by 25 ± 6%, although the cell number was not suppressed significantly.

Percutaneous absorption and penetration of VC-PMG into human skin

Permeation of ^{14}C-labeled VC-PMG was measured and assessed to determine the in vitro percutaneous absorption and penetration through human skin (Table IV). We measured (1) VC-PMG absorption into the epidermis and dermis, (2) VC-PMG removed from the skin surface by washing, and (3) VC-PMG penetrated through the skin and into the receptor phase. In addition, the percentage of the applied dose recovered was calculated. The percutaneous penetration of the radiolabeled VC-PMG was low, ranging from 0.09% to 0.51% of the applied dose. The amount of radiolabeled product in the entire skin after topical application of the cream was 1.58%, obtained by the addition of the amount found in the epidermis and dermis. The amount of radiolabeled product in the skin was changed by the ad-

Fig. 1. A patient with chloasma, before treatment.

Fig. 2. Same patient as in Fig. 1 after 3 months of treatment.

dition of 1,1-methyleneglycol-bis to the cream vehicle. The highest absorption and penetration into the epidermal and dermal levels were observed with the formulations containing 1%, 1,1-methyleneglycol-bis and 3%,1,1-methyleneglycol-bis, respectively.

Lightening effects of VC-PMG on pigmentation

The VC-PMG cream was effective or fairly effective in 19 of 34 patients (Table V). VC-PMG cream was also applied to normally pigmented healthy skin of 25 patients. The results were effective in one, fairly effective in two, slightly effective in eight, not effective in 12, and possible darkening in two. Fig. 1 shows a patient with chloasma before treatment; VC-PMG was applied to the entire face. After 3 months of treatment, the pigmentation lightened (Fig. 2).

DISCUSSION

The topical application of VC-PMG was effective in lightening the skin of some patients with hyperpigmentation disorders and some subjects with normally pigmented healthy skin. Our results suggest that inhibition of melanogenesis was stronger when the activity of melanogenic enzymes was relatively high. It has been proposed that the influence of AsA on the monopherase activity of tyrosinase is from its ability to reduce the enzymatically generated *o*-quinones. In this study, we measured the effect of VC-PMG on purified tyrosinase, B16F10 cell extract, and cultured human melanoma cells. We conclude that VC-PMG directly or indirectly suppresses melanin formation catalyzed by mammalian tyrosinase. TRP1 had been suggested to have a variety of catalytic functions, including low levels of those enzymatic activities ascribed to tyrosinase.[16, 17] TRP1 has also been reported to possess 5,6-dihydroxyindole-2-carboxylic acid oxidase activity, an activity blocked by AsA.[32] AsA probably suppresses melanin formation at various oxidative steps of melanin formation, such as 5,6-dihydroxyindole oxidation.

The disadvantage of AsA is its instability, especially in aqueous solution. However, VC-PMG significantly suppressed melanin formation on purified tyrosinase or cultured cells and inhibited melanin formation without cell growth suppression on cultured human melanoma cells. VC-PMG has been assumed to have lower vitamin C activity than AsA.[33] However, in this study, 10^{-3}% (approximately 2.6×10^{-5} mol/L) VC-PMG suppressed melanin formation by mammalian tyrosinase by 33%. Differences in the methods of assay or differences in the tyrosinases may account for these discrepancies.

Application of 10% VC-PMG cream to the skin

of patients with hyperpigmented disorders, such as ephelides, chloasma, or senile freckles lightened the pigmentation in some. Furthermore, VC-PMG also produced the same lightening in normally pigmented healthy skin. These results suggest that VC-PMG is absorbed percutaneously, stays in the skin, and inhibits tyrosinase activity of melanocytes. The in vitro percutaneous absorption data also suggest that the addition of 1% to 3% 1,1-methyleneglycol-bis increases the absorption of VC-PMG.

REFERENCES

1. Mumma RO, McKee EE, Verlangieri AJ, et al. Anti-ascorbutic effect of ascorbic acid 2-sulfate in the guinea pig. Nutr Rep Int 1972;6:133-7.
2. Manning JM, Meister A. Conversion of proline to collagen hydroxyproline. Biochemistry 1966;5:1154-65.
3. Phillips CL, Combs SB, Pinnell SR. Effects of ascorbic acid on proliferation and collagen synthesis in relation to the donor age of human dermal fibroblasts. J Invest Dermatol 1994;103:228-32.
4. Zannoni VG, Sato PH. The effect of certain vitamin deficiencies on hepatic drug metabolism. Fed Am Soc Exp Biol 1976;35:2464-9.
5. Dahl H, Degré M. The effect of ascorbic acid on production of human interferon and the antiviral activity in vitro. Acta Pathol Microbiol Scand Sect B 1976;84:280-4.
6. Salo RJ, Cliver DO. Inactivation of enteroviruses by ascorbic acid and sodium bisulfite. Appl Environ Microbiol 1978;36:68-75.
7. Rawal BD, Mckay G. Inhibition of *Pseudomonas aeruginosa* by ascorbic acid acting singly and in combination with antimicrobials: in-vitro and in-vivo studies. Med J Aust 1974;1:169-74.
8. Bielski BHJ, Richter HW. Some properties of the ascorbate free radical. Ann N Y Acad Sci 1975;258:231-7.
9. Meister A. Glutathione-ascorbic acid antioxidant system in animals. J Biol Chem 1994;269:9397-400.
10. Hearing VJ, Tsukamoto K. Enzymatic control of pigmentation in mammals. FASEB J 1991;5:2902-9.
11. Pawelek JM. After DOPAchrome. Pigment Cell Res 1991; 4:53-62.
12. Körner AM, Pawelek J. Mammalian tyrosinase catalyzes three reactions in the biosynthesis of melanin. Science 1982;217:1163-5.
13. Kwon BS, Halaban R, Kim GS, et al. Melanocyte specific complementary DNA clone whose expression is inducible by melanotropin and isobutylmethyl xanthine. Mol Biol Med 1987;4:339-55.
14. Kwon BS, Jak AK, Pomerantz SH, et al. Isolation and sequence of a cDNA clone for human tyrosinase that maps at the mouse c-albino locus. Proc Natl Acad Sci U S A 1987;84:7473-7.
15. Barton DE, Kwon BS, Franke U. Human tyrosinase gene mapped to chromosome 11 (q14-q21), defines second region of homology with mouse chromosome 7. Genomics 1988;3:17-24.
16. Jiménez M, Maloy WL, Hearing VJ. Specific identification of authentic clone for mammalian tyrosinase. J Biol Chem 1989;264:3397-403.
17. Jiménez M, Tsukamoto K, Hearing VJ. Tyrosinases from two different loci are expressed by normal and by transformed melanocytes. J Biol Chem 1991;266:1147-56.
18. Tsukamoto K, Jackson IJ, Urabe K, et al. A second tyrosinase-related protein, TRP-2, is a melanogenic enzyme termed DOPAchrome tautomerase. EMBO J 1992;11:519-26.
19. Yokoyama K, Suzuki H, Yasumoto K, et al. Molecular cloning and functional analysis of a cDNA coding for human DOPAchrome tautomerase/tyrosinase-related protein-2. Biochim Biophys Acta 1994;1217:317-21.
20. Kameyama K, Jiménez M, Muller J, et al. Regulation of mammalian melanogenesis by tyrosinase inhibition. Differentiation 1989;42:28-36.
21. Kameyama K, Takemura T, Hamada Y, et al. Pigment production in murine melanoma cells is regulated by tyrosinase, tyrosinase-related protein 1 (TRP1), DOPAchrome tautomerase (TRP2), and a melanogenic inhibitor. J Invest Dermatol 1993;100:126-31.
22. Lerner AB, Fitzpatrick TB. Biochemistry of melanin formation. Physiol Rev 1950;30:91-126.
23. Ros JR, Rodriguez-Lopes JN, Garcia-Canovas F. Effect of L-ascorbic acid on the monophelase activity of tyrosinase. Biochem J 1993;295:309-12.
24. Nomura H, Ishiguro T, Morimoto S. Studies on L-ascorbic acid derivatives. II. L-Ascorbic acid 3-phosphate and 3-pyrophosphate. Chem Pharm Bull (Tokyo) 1989;17:381-6.
25. Lee CH, Seib PA, Liang YT, et al. Chemical synthesis of several phosphoric esters of L-ascorbic acid. Carbohydr Res 1978;67:127-38.
26. Mima H, Nomura H, Imai Y, et al. Chemistry and application of ascorbic acid phosphate. Vitamins (Japan) 1970; 41:387-98. (In Japanese)
27. Kameyama K, Takezaki S, Kanzaki T, et al. HLA-DR and melanoma-associated antigen (p97) expression during the cell cycle in human melanoma cell lines, and the effects of recombinant gamma-interferon: two-color flow cytometric analysis. J Invest Dermatol 1986;87:313-8.
28. Kameyama K, Vieira WD, Tsukamoto K, et al. Differentiation and the tumorigenic and metastatic phenotype of murine melanoma cells. Int J Cancer 1990;45:1151-8.
29. Kameyama K, Montague PM, Hearing VJ. Expression of melanocyte stimulating hormone receptors correlates with mammalian pigmentation, and can be modulated by interferons. J Cell Physiol 1988;137:35-44.
30. Hearing VJ. Mammalian monophenol monooxygenase (tyrosinase): purification, properties, and reactions catalyzed. In: Kaufman S, ed. Methods in enzymology; vol 142. New York: Academic Press, 1987:154-65.
31. Bronaugh RL, Stewart RF. Methods for in vitro percutaneous absorption studies. IV. The flow-through diffusion cell. J Pharm Sci 1985;74:64-7.
32. Jiménez-Cervantes C, Solano F, Kobayashi T, et al. A new enzymatic function in the melanogenic pathway. J Biol Chem 1994;269:17993-8001.
33. Takashima H, Nomura H, Imai Y. Ascorbic acid esters and skin pigmentation. Am Perfumer Cosmet 1971;86:29-36.