Q26. I have examined patients who believe that depigmentation may have been caused by other cosmetics. What is the situation regarding this?

A26. There have been a few reports of similar depigmentation occurring because of the use of skin-whitening cosmetics other than those containing Rhododenol. The Ministry of Health, Labor and Welfare is investigating whether any notifications have been received by cosmetics companies from dermatologists. Regarding the diagnostic criteria for a medical institution to provide information to a company, there is a legal obligation to report serious cases (including patients who require treatment for ≥30 days) to companies if the possibility of a side effect of a cosmetic or other product cannot be ruled out; this should be used as a guideline for when to provide information. However, it should be noted that medical institutions are not necessarily obliged to report cases to corporations.

If the manufacturer's contact details are unknown, the matter may be reported directly to the Pharmaceuticals and Medical Devices Agency (PMDA) as a patient safety report; the PMDA may request the company to both provide information and instruct it to gather additional information if necessary. In this process, record the patient's responses to the medical interview and findings on clinical examination in accordance with the "Questionnaire on Cases of Depigmentation due to the Use of Cosmetics Not Containing Rhododenol". This questionnaire also includes sections for information on tests and treatment, and it is designed so as not to interfere with patient examinations. The Special Committee is not engaged in investigating depigmentation that develops after the use of cosmetics that do not contain Rhododenol or the elucidation of its pathogenesis.

Members of the Japanese Society for Dermatoallerology and Contact Dermatitis are gathering information via the Case Information Network of Skin Safety on Cosmetics and Other Products (http://jsac-public.sharepoint.com/). We would be grateful for cooperation with this network when submitting a report to a manufacturer or the PMDA.

The JDA website contains a report from the Ministry of Health, Labor and Welfare on leukoderma and other skin disorders developed on users of topical quasi-medications and cosmetics other than cosmoceuticals containing Rhododenol (https://www.dermatol.or.jp/modules/guideline/index.php?content_id=8). Please see this article for more information.

CONFLICT OF INTEREST: None.

APPENDIX 1

PRIMARY QUESTIONNAIRE (TO BE COMPLETED BY DOCTOR)

Name of Institution _____

Address of Institution _____ Tel: _____

Date Completed (mm/dd/yyyy) / /20 Name of Doctor _____

E-mail: _____

□Patient attributes

Patient ID: Age: years Sex Female/Male

□Occupational history Current occupation: Housewife/Company employee/Other ( )

Previous exposure to phenol/phenol compounds (Note 1): No/Yes/Unknown

□Family history: Vitiligo vulgaris: No/Yes

Other family history: No/Yes ( )

□Previous medical history: Vitiligo vulgaris: No/Yes Hay fever: No/Yes (in which month(s)?)

Hives: No/Yes (caused by:) Allergic dermatitis: No/Yes

Contact dermatitis: No/Yes (caused by:) Asthma: No/Yes

Alopecia: No/Yes Diabetes: No/Yes (Type I/Type II)

Psoriasis vulgaris: No/Yes Addison's disease: No/Yes

Pityriasis versicolor: No/Yes Connective tissue disease: No/Yes ( )

Thyroid disorder: No/Yes (if yes, name of disorder)

History of use of medications that may induce melanoleukoderma: No/Yes

□History of previous cosmetic use

1) Hydroquinone products: No/Yes (hydroquinone concentration %, period of use from (year) to (year))

→If Yes, were there any skin problems? No/Yes

2) Skin-whitening cosmetics other than Rhododenol No/Yes (Product name: , period of use from (year) to (year))

3) History of use of hair dyes: No/Yes (period of use since years ago, frequency once every)

→If Yes, were there any skin problems? No/Yes

4) History of use of hair growth promoters: No/Yes (product name: , period of use from (year) to (year))

→If Yes, were there any skin problems? No/Yes

□Photographs

1) Clinical photographs of areas of pigment loss: No/Yes

2) Dermoscopic photographs of areas of pigment loss: No/Yes

3) Clinical photographs of erythema or other areas of inflammation: No/Yes

4) Clinical photographs of areas of pigment deposition: No/Yes

5) Permission to submit photographs to the Special Committee: No/Yes

*Please obtain the patient's permission to submit photographs.

□Clinical symptoms

1) Month of onset: /20 (age years) (as reported by patient)
2) Type of pigment loss (clinical): total leukoderma/incomplete leukoderma/mixed type

3) Site(s) of pigment loss: face/neck/backs of hands/forearms/upper arms/other

( )

4) Area of pigment loss: Face: approximately 0–25%/approximately 25–50%/almost entire area

Neck: approximately 0–25%/approximately 25–50%/almost entire area

Backs of hands: approximately 0–25%/approximately 25–50%/almost entire area

Forearms: approximately 0–25%/approximately 25–50%/almost entire area

Upper arms: approximately 0–25%/approximately 25–50%/almost entire area

5) Site(s) where the product was used: face/neck/backs of hands/forearms/upper arms/other ( )

6) Inflammatory symptoms such as erythema, itching, or scaliness at sites where the product was used: No/Yes

7) Coincidence between sites of use and sites of inflammation: Mostly consistent/Not consistent

→If not consistent: inflammation in areas where the product was not applied/Widespread inflammatory symptoms from the margins of areas where the product was applied

8) Coincidence between areas where the product was applied and areas of pigment loss: Mostly consistent/Not consistent

→If Not consistent: Pigment loss in areas where the product was not applied/Widespread pigment loss from the margins of areas where the product was applied

9) Pigment loss from areas where the product has not been used: No/Yes (area:)

10) Photosensitivity (clinical, subjective on the part of the patient): No/Yes/Unknown

11) Can be diagnosed as vitiligo vulgaris: No/Yes/Indeterminate

12) Pigment deposition: No/Yes

13) Mixed pigment loss and pigment deposition (sign of melanoleukoderma): No/Yes

□Tests

1) Blood tests: No/Yes (if possible, measure antinuclear antibodies, anti-thyroglobulin antibody, anti-peroxidase antibody, TSH, FT3, and FT4)

2) Skin biopsy: No/Yes/Scheduled (scheduled date)

3) Patch test: No/Yes Scheduled (scheduled date)

4) MED measurement: No/Yes →UVA MRD J/cm^2 Contraction: No/Yes

UVB MED mJ/cm^2 Contraction: No/Yes

□Treatment: No (monitoring only) Yes (name of drug:)

□Course

1) Time since discontinuing use of cosmetics containing Rhododenol: years months

2) Follow-up period at this institution: years months

3) Status of pigment loss: Recovered/Improved/No change/Worse

Note 1: These chemicals are found in adhesives, inks, varnishes, various types of synthetic resin-modifying agents, raw materials for perfumes, insecticides, herbicides, rubber antioxidants, raw materials for vinyl chloride stabilizers, surfactants and other antioxidants, and oil additives.

Note 2: Drugs that may induce melanoleukoderma

Thiazide antidiuretics	Hydrochlorothiazide, chlorothiazide
Other antidiuretics	meticrane
Antibiotics	tetracycline, fleroxacin, griseofulvin
Muscle relaxants	afloqualone
Nonsteroidal anti-inflammatories	tiaprofenic acid
Beta-blockers	pindolol

APPENDIX 2

SECONDARY QUESTIONNAIRE ON RHODODENOL-INDUCED LEUKODERMA

Name of Institution _____ Address of Institution _____

Age: years Sex: Female/Male

1) Did you previously send a primary questionnaire for this patient to the Secretariat of the Japan Dermatological Association?

( ) Primary questionnaire sent → Institutional registration number of primary questionnaire (_____)

( ) Primary questionnaire not sent (*secondary questionnaires may also be submitted for patients for which primary questionnaires have not been sent)

Institutional registration number (_____)

2) Status of depigmentation on initial examination

(a) Initial examination: Which of the following out of Kanebo's classification applies?

1 ( ) Obvious vitiligo across a wide area of the face, neck, hands, or elsewhere

2 ( ) "Vitiligo in at least three places," "vitiligo at least 5 cm in size," or "obvious vitiligo on the face"

3 ( ) None of the above apply

(b) On initial examination, what was the depigmentation score according to the score sheet attached as Appendix 3

1 ( ) points on initial examination 2 ( ) Could not be evaluated on initial examination 3 Other ( )

↓ Fill in the items below only for patients whose condition is being monitored.

3) In terms of the diagnostic criteria listed in Attachment 2, which of the following applies to this patient?

1 ( ) Definite case 2 ( ) Suspected case

4) Which of the clinical types described in Attachment 2 applies to this patient?

1 ( ) Predominantly total leukoderma 2 ( ) Mixed total and incomplete leukoderma 3 ( ) Predominantly incomplete leukoderma

5) When this questionnaire was completed, how much time had elapsed since monitoring started at your institution and the patient discontinued using cosmetics containing Rhododenol? (Fill in the number of months, with 1 year = 12 months)

- Duration of monitoring at your hospital or clinic: months
- Time since the patient discontinued using cosmetics containing Rhododenol: months

6) Have you treated the patient while they were being monitored at your institution?

1 ( ) Yes 2 ( ) No

7) If Yes, please indicate the type of treatment.

- Oral medication: ( ) No ( ) Yes: Name of medication _____
- Topical medication: ( ) No ( ) Yes: Name of medication _____
- Ultraviolet light: ( ) No ( ) Yes ( ) Eximer ( ) NB-UVB

Other ultraviolet light ( )

- If any other type of treatment has been administered, please describe it below

_____

8) Describe the course of symptoms

(a) Color of unaffected areas (surrounding areas of depigmentation, healthy areas)

1 ( ) Hyperpigmentation is evident with no return to normal skin color as of yet

2 ( ) Transient hyperpigmentation was evident, but skin color has since returned to normal

3 ( ) Pigment has regenerated with no intensification

(b) Area of depigmentation

1 ( ) The area of depigmentation is growing

2 ( ) The area of depigmentation is unchanged

3 ( ) The area of depigmentation has decreased but remains more than half its initial size

4 ( ) The area of depigmentation is between around one-quarter and one-half of its initial size

5 ( ) The area of depigmentation is less than one-quarter of its initial size

6 ( ) The depigmentation has almost entirely disappeared

9) Evaluation of the course of symptoms on the basis of an overall assessment including areas of depigmentation, hyperpigmentation, and the patient's perception (i.e., psychological stress)

1 ( ) Recovered 2 ( ) Greatly improved 3 ( ) Improved 4 ( ) Somewhat improved 5 ( ) No change 6 ( ) Worse

10) If it has been possible to evaluate depigmentation in this patient at 1, 3, and 6 months or longer after discontinuing use of cosmetics containing Rhododenol, please fill in the table below.

	Kanebo classification	Depigmentation score
	1: Obvious vitiligo across a wide area of the face, neck, hands, or elsewhere 2: Either "vitiligo in at least three places," "vitiligo at least 5 cm in size," or "obvious vitiligo on the face" 3: None of the above apply	* See the attachment to assess the depigmentation score. +S: Add to the end of the score if depigmentation has developed at sites where the cosmetics concerned were not used and is spreading. +α: Add to the end of the score if depigmentation has developed in a location other than those listed as 1–6.
1 month after initial examination	Kanebo classification ( )/Could not be evaluated	Score ( )/Could not be evaluated
3 months after initial examination	Kanebo classification ( )/Could not be evaluated	Score ( )/Could not be evaluated
6 months after initial examination	Kanebo classification ( )/Could not be evaluated	Score ( )/Could not be evaluated
( ) months after initial examination	Kanebo classification ( )	Score ( )

11) Please feel free to give your opinions or describe any specific features of this case below.

APPENDIX 3

DEPIGMENTATION SCORE SHEET

*This sheet need not be returned with the secondary questionnaire.
Patient ID _____ Date (mm/dd/yyyy) / / ____ Evaluator:

A Location	B Assessment of whiteness of depigmentation	C Extent of depigmentation	D Subtotal (B × C)
	No depigmentation 0	0% 0	
	Incomplete leukoderma 1	1–25% 1	
	Complete leukoderma 2	26–50% 2	
		50–75% 3	
		76–100% 4	
	Score 2 if mixed incomplete and complete leukoderma are present	Area of leukoderma/total area evaluated	
1 Forehead			
2 Eyebrows/Upper and lower eyelids/External canthi			
3 Cheeks			
4 Nose/Around the mouth			
5 Lower jaw/Front of the neck/Side of the neck			
6 Backs of the hands/Between the fingers			
Other locations ( ) ( ) ( ) ( )		Total depigmentation score (If necessary, add +α or +S to the end of the score)	

Note 1: The depigmentation score is calculated as sum of all the scores assessed for areas 1–6 above.
Note 2: If depigmentation has developed at locations other than those listed in 1–6 above, add +α to the end of the total score (e.g. 24 + α).
Note 3: If depigmentation has developed at sites where the cosmetics concerned were not used and is spreading, add +S to the end of the total score (e.g. 24 + S or 24 + α + S).
Note 4: If depigmentation has developed in an area other than those listed in 1–6 above, fill in the area concerned in the "Other locations" section.

Pigment Cell Melanoma Res. 27; 754–763

ORIGINAL ARTICLE

Rhododendrol, a depigmentation-inducing phenolic compound, exerts melanocyte cytotoxicity via a tyrosinase-dependent mechanism

Minoru Sasaki[1], Masatoshi Kondo[1], Kohji Sato[1], Mai Umeda[1], Keigo Kawabata[1], Yoshito Takahashi[1], Tamio Suzuki[2], Kayoko Matsunaga[3] and Shintaro Inoue[1]

1 Innovative Beauty Science Laboratory, Kanebo Cosmetics Inc., Kanagawa, Japan **2** Department of Dermatology, Yamagata University School of Medicine, Yamagata, Japan **3** Department of Dermatology, Fujita Health University School of Medicine, Toyoake, Japan

CORRESPONDENCE Shintaro Inoue, e-mail: inoue.shintarou@kanebocos.co.jp

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Summary

Rhododendrol, an inhibitor of melanin synthesis developed for lightening/whitening cosmetics, was recently reported to induce a depigmentary disorder principally at the sites of repeated chemical contact. Rhododendrol competitively inhibited mushroom tyrosinase and served as a good substrate, while it also showed cytotoxicity against cultured human melanocytes at high concentrations sufficient for inhibiting tyrosinase. The cytotoxicity was abolished by phenylthiourea, a chelator of the copper ions at the active site, and by specific knockdown of tyrosinase with siRNA. Hence, the cytotoxicity appeared to be triggered by the enzymatic conversion of rhododendrol to active product(s). No reactive oxygen species were detected in the treated melanocytes, but up-regulation of the CCAAT-enhancer-binding protein homologous protein gene responsible for apoptosis and/or autophagy and caspase-3 activation were found to be tyrosinase dependent. These results suggest that a tyrosinase-dependent accumulation of ER stress and/or activation of the apoptotic pathway may contribute to the melanocyte cytotoxicity.

Introduction

Rhododendrol (4-(4-hydroxyphenyl)-2-butanol, Rhododenol®), a naturally occurring phenolic compound in plants such as *Acer nikoense* and *Betula platyphylla* (Fuchino et al., 1996; Inoue et al., 1978), was developed as a tyrosinase inhibitor for lightening/whitening cosmetics (Figure 1). Products containing a 2% (w/w) formulation of rhododendrol were available on the Japanese market for about 5 yr. These products, however, were recently withdrawn from the market after rhododendrol was reported to cause a depigmentary disorder (The Japanese Dermatological Association Special Committee on the Safety of Cosmetics Containing Rhododenol, 2014,

Significance

A rhododendrol-induced leukoderma has recently been reported in Japan. Rhododendrol is a naturally occurring phenolic compound developed for lightening/whitening cosmetics. The etiology of this leukoderma requires urgent clarification, as the clinical and pathological details are still largely unknown. Our results demonstrated that rhododendrol competitively inhibited mushroom tyrosinase and served as a good substrate. Findings obtained with tyrosinase siRNA clearly indicated that rhododendrol exerts tyrosinase-dependent melanocyte cytotoxicity with a concomitant induction of ER stress and apoptosis. These findings may help us understand similar types of cutaneous depigmentation, such as chemical leukoderma and idiopathic vitiligo.

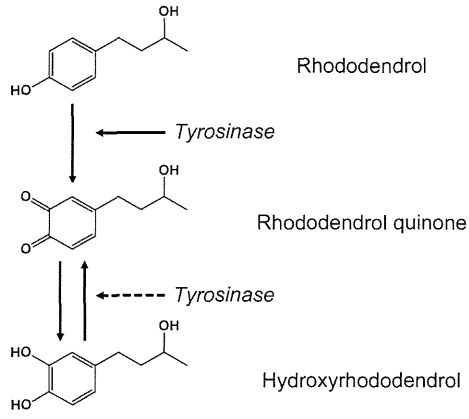

Figure 1. Outline of putative metabolic pathway involved in generation of hydroxyrhododendrol or *o*-quinone from rhododendrol.

(in Japanese)). As of about 6 months after the announcement, the symptom of depigmentation had been confirmed in about 16 000 (2%) of 800 000 estimated users of cosmetic products containing rhododendrol. The symptom was observed principally at the sites of repeated contact with the chemical, and 79% of the affected persons who had discontinued use for 6 months showed a trend toward improvement. The etiology of this rhododendrol-induced leukoderma requires urgent clarification, as the clinical and pathological details are still largely unknown.

Chemical leukoderma is defined as an acquired hypopigmentation caused by repeated exposure to specific agents damaging to epidermal melanocytes. The condition may develop at the sites of contact with the instigating chemicals or remotely from the exposure (Ghosh, 2010). No absolute clinicohistopathological criteria can differentiate chemical leukoderma from vitiligo, an acquired chronic disorder characterized by skin depigmentation due to localized loss of epidermal melanocytes. The etiology of vitiligo is only partially understood, but genetic, immunological, and environmental factors are all thought to take part in the pathogenesis. Oxidative stress may trigger melanocyte damage in individuals susceptible to vitiligo, but the triggers in most cases diagnosed with idiopathic vitiligo are unknown (Boissy and Manga, 2004). Chemical leukoderma is distinct from the vitiliginous process seen in occupational/chemical vitiligo, which switches on initially in response to chemicals but continues even after the chemical exposure ceases (Boissy and Manga, 2004; Cummings and Nordlund, 1995). The causative chemicals are mostly aromatic or aliphatic derivatives of phenols and catechols, but many other chemicals are capable of conferring similar depigmenting effects, such as sulfhydryls, mercurials, arsenics, cinnamic aldehyde, *p*-phenylenediamine, benzyl alcohol, azelaic acid, corticosteroids, eserine, thiotepa, chloroquine, and fluphenazine. Yet, none of these chemical triggers of depigmentation appear to be lethal for melanocytes in individuals without specific inherent susceptibilities (Boissy and Manga, 2004).

Monobenzyl ether of hydroquinone (MBEH) and 4-tert-butyl phenol (4-TBP) are phenolic compounds widely known to initiate a disease indistinguishable from idiopathic vitiligo (Boissy and Manga, 2004). Patients exposed to MBEH generally undergo a permanent depigmentation consistent with a total removal of melanocytes (Bolognia et al., 2001), though pigment may return in some individuals (Oakley, 1996). The compound 4-TBP causes occupational vitiligo in individuals working in the rubber and tannery industries (James et al., 1977), and appears to be specifically cytotoxic to melanocytes (Yang and Boissy, 1999). The working mechanism of these phenolic compounds remains obscure, but both compounds have been hypothesized to act as substrates for tyrosinase, the rate-limiting enzyme for melanogenesis, due to their structural similarity to tyrosine. A catalytic action of melanocyte tyrosinase is believed to lead to the generation of reactive *o*-quinone radicals from phenolic compounds, the induction of cellular oxidative stress, and the cytotoxicity. Yet according to the original studies, MBEH and 4-TBP become cytotoxic to the melanocytes via different pathways. Specifically, MBEH induces a non-apoptotic cell death through a necrotic pathway, while 4-TBP triggers apoptosis (Hariharan et al., 2010). MBEH was also found to up-regulate the levels of melanogenic enzymes in cultured melanocytes and skin explants, whereas 4-TBP reduced them. Also, though 4-TBP is a tyrosine analog that acts as a competitive inhibitor, its cytotoxicity is not linked to tyrosinase activity, but rather the levels of tyrosinase-related protein 1 (TRP-1) and microphthalmia-associated transcription factor (Manga et al., 2006). Toosi et al. (2012) recently reported that MBEH and 4-TBP caused oxidative stress in melanocytes, thereby initiating ER stress and activation of the unfolded protein response (UPR) with interleukin (IL)-6 and 8 production, processes that may play a critical role in melanocyte viability. These studies indicate that the working mechanisms of the phenolic chemicals for melanocyte cytotoxicity involve factors more complicated than the tyrosine analogs for the tyrosinase.

In this study, we examined the effects of rhododendrol on cultured human melanocytes to understand the possible mechanisms of rhododendrol-induced leukoderma caused by repeated contact with the phenolic compound. Our findings clearly revealed a tyrosinase-dependent cytotoxicity of rhododendrol against melanocytes with a tyrosinase-dependent induction of ER stress and activation of the apoptotic pathway. These findings may help to explain similar forms of chemically induced cutaneous depigmentation such as chemical leukoderma and idiopathic vitiligo.

Results

Rhododendrol suppresses tyrosinase activity of cultured human melanocytes and inhibits mushroom tyrosinase competitively

When rhododendrol was added to cultured human melanocytes, cellular tyrosinase activity was dose

dependently suppressed with an IC_{50} value of 5.3 μM, whereas rhododendrol had no effects on the cellular protein synthesis at concentrations examined (Figure 2A). Rhododendrol also inhibited melanin synthesis of mouse B16 melanoma cells at the same range of concentrations (Figure S1). A Lineweaver–Burk plot analysis showed that rhododendrol inhibited mushroom tyrosinase competitively. The apparent Km value of tyrosinase for L-tyrosine was 0.36 mM (Figure 2B), which is comparable with the Km value for L-tyrosine (0.4 mM) reported by Pomerantz (1966).

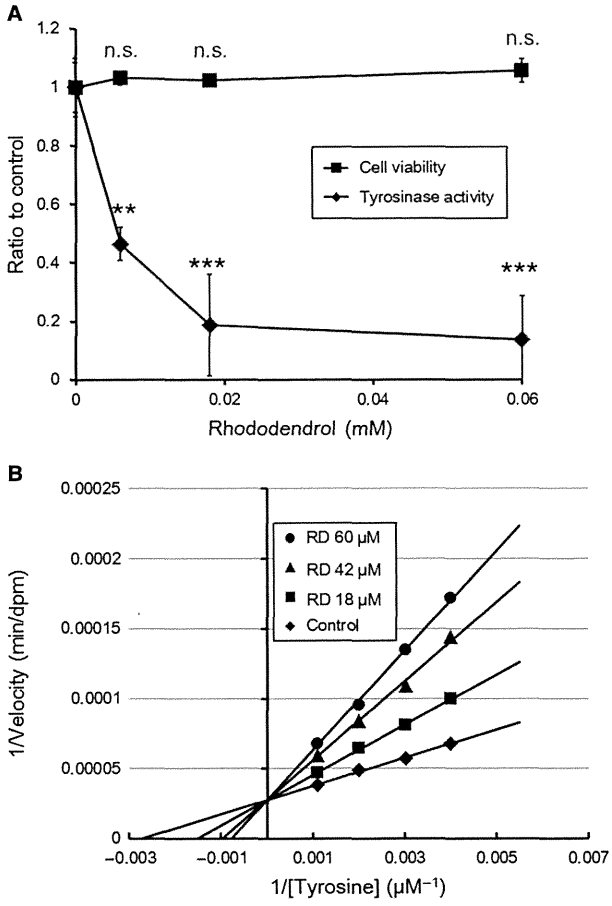

Figure 2. Rhododendrol inhibits tyrosinase when estimated with L-tyrosine as a substrate. (A) Tyrosine hydroxylase activity and cell viability estimated by protein synthesis were evaluated in cultured human melanocytes treated with various concentrations of rhododendrol. Results are expressed as mean ± SD of triplicate experiments (**P < 0.01, ***P < 0.001 vs. 0 mM, Tukey's test). (B) A Lineweaver–Burk plot of tyrosinase using tyrosine as a substrate reveals a competitive inhibition of mushroom tyrosinase by rhododendrol (RD), with an apparent Km value of 0.36 mM. Twenty units of mushroom tyrosinase were incubated in 500 μl of PBS containing 0.1 mM DOPA, 0.25–0.9 mM of tyrosine as a substrate with a constant proportion of radiolabeled tyrosine, and 3–10 μg/ml of rhododendrol at 37°C for 5 min. Velocity is shown as radioactivity (dpm) per reaction time which is written in Methods section. Each experiment was performed in triplicate.

Rhododendrol competed with L-tyrosine for mushroom tyrosinase, with a Ki value of 24 μM as determined by a Dixon plot analysis (Figure S2).

Rhododendrol serves as a good substrate for mushroom tyrosinase

As rhododendrol is a phenolic tyrosine analog, we examined the possibility that mushroom tyrosinase catalyzes rhododendrol as a substrate. The production of tritiated water from 3′, 5′-[^{3}H]-rhododendrol indicated that rhododendrol can be catalyzed by the tyrosinase. Rhododendrol served as a good substrate in place of L-tyrosine according to the Lineweaver–Burk plot analysis, with an apparent Km of 0.27 mM (Figure 3), a value comparable with the Km for L-tyrosine (Km = 0.36 mM) (Figure 2B).

Tyrosinase activity is essential for rhododendrol cytotoxicity

We hypothesized that the cytotoxicity induced by rhododendrol is dependent on cellular tyrosinase activity, as rhododendrol can be catalyzed by tyrosinase. We tried to confirm this hypothesis by inhibiting tyrosinase activity using phenylthiourea (PTU), a chelator of the copper ions necessary for tyrosinase activity (Ryazanova et al., 2012). Rhododendrol hydroxylase activity was dose dependently suppressed by PTU, with no marked cytotoxicity (Figure 4A). Treatment with PTU at concentrations of 10 to 100 μM attenuated rhododendrol-induced cytotoxicity in a dose-dependent manner (Figure 4B). Identical tyrosinase-dependent cytotoxicity of rhododendrol was also found in other human melanocyte strains and mouse B16 melanoma cells (Figure S3).

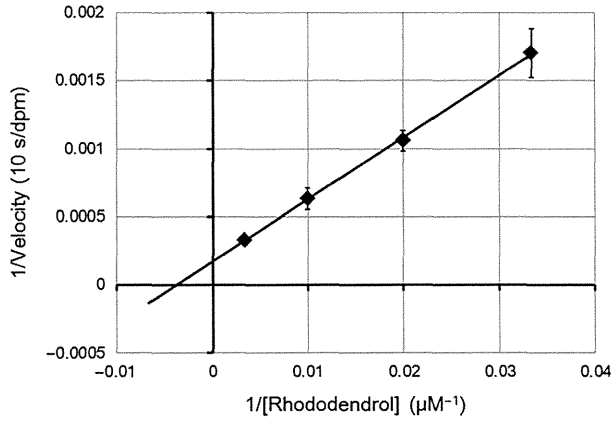

Figure 3. Lineweaver–Burk plot of tyrosinase using rhododendrol as a substrate. Ten units of mushroom tyrosinase was incubated in 500 μl of PBS containing 1.0 mM DOPA and 0.03–0.3 mM of rhododendrol with a constant proportion of radiolabeled rhododendrol at 37°C for 10 sec. The inverse of the X-intercept of the regression line indicates an apparent Km value of 0.27 mM. Results are expressed as mean ± SD of triplicate experiments. Velocity is shown as radioactivity (dpm) per reaction time which is written in Methods section.

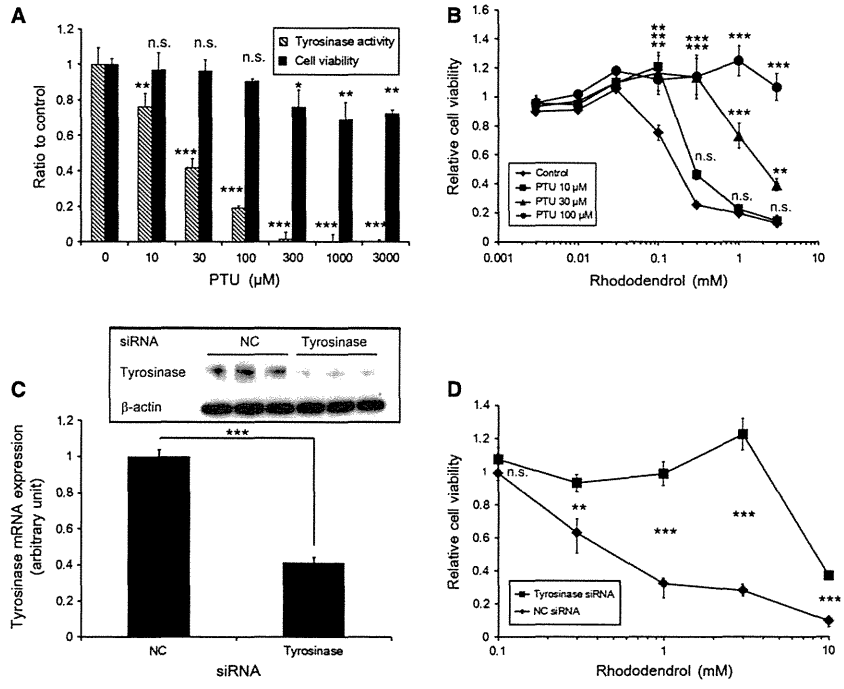

Figure 4. Tyrosinase activity is required for the melanocyte cytotoxicity induced by rhododendrol. (A) Cultured melanocytes were treated with phenylthiourea (PTU), an agent that inhibits tyrosinase via chelation of the copper ions, in order to examine the effects on rhododendrol hydroxylase activity and cell viability. Cells were treated with various concentrations of PTU (10–1000 μM) for 24 h and then further incubated for 24 h with 1 μCi of [^{3}H]-rhododendrol per well. At the end of the incubation, cell viability was assessed by alamarBlue assay (*P < 0.05, **P < 0.01, ***P < 0.001 vs. 0 mM, Tukey's test). (B) Cultured human melanocytes were pre-treated with PTU at various concentrations (10–100 μM) and then treated with rhododendrol for 24 h. Cell viability was assessed by alamarBlue assay (**P < 0.01, ***P < 0.001 vs. non-treated, Tukey's test). (C) Cultured human melanocytes were transfected with siRNA against tyrosinase and a negative control (NC) siRNA, as described in the Methods section. Tyrosinase mRNA was determined by real-time PCR with the normalization by GAPDH mRNA. Tyrosinase protein expression was analyzed by immunoblotting with β-actin as an internal control (***P < 0.001, Student's *t*-test). (D) The effect of tyrosinase knockdown on the cytotoxicity induced by various concentrations (0.1–10 mM) of rhododendrol. Cell viability was assessed by alamarBlue assay (**P < 0.01, ***P < 0.001, Student's *t*-test). Results are expressed as mean ± SD of triplicate experiments.

Next, we investigated whether the specific knockdown of tyrosinase expression could result in the same attenuation of cytotoxicity as PTU treatment. The knockdown efficacy of siRNA against tyrosinase mRNA in melanocytes was about 60%. In contrast to the negative control siRNA, the tyrosinase siRNA elicited a significant down-regulation of tyrosinase in protein expressions (Figure 4C). The depletion of tyrosinase almost completely rescued cells from damage by rhododendrol at concentrations as high as 3 mM (Figure 4D). These findings indicated that the metabolism of rhododendrol by tyrosinase is required for rhododendrol cytotoxicity. In contrast, the cell damage induced by 10 mM rhododendrol appeared to be a non-specific cell death caused by high concentration exposure to chemicals, as the tyrosinase depletion failed to rescue the cells (Figure 4D). At lower concentrations, rhododendrol increased cell viability rather than inducing cytotoxicity (Figure 4B and D).

Hydroxyrhododendrol is more toxic than rhododendrol

Noting that the expression of rhododendrol cytotoxicity seemed to require tyrosinase activity, we decided to compare the cytotoxic potential of hydroxyrhododendrol, a putative metabolic product of rhododendrol, with that of rhododendrol, because rhododendrol quinones are too unstable to characterize such toxicity (Figure 1) (Cooksey et al., 1997). Hydroxyrhododendrol at concentrations above 0.1 mM eradicated nearly all of the cells (IC_{50} = 0.06 mM), while cells treated with the same concentrations of rhododendrol survived (Figure 5). Hydroxyrhododendrol is more toxic than rhododendrol.

Rhododendrol induces no detectable reactive oxygen species (ROS) in human melanocytes

As hydroxyrhododendrol-derived quinone radicals are believed be involved in cytotoxicity, we tried to detect the extent of reactive oxygen species (ROS) generation induced by rhododendrol and hydroxyrhododendrol treatments in human melanocytes. After 3 h of treatment with rhododendrol or hydroxyrhododendrol, a dose-dependent up-regulation of ROS was detected in melanocytes treated with hydroxyrhododendrol above concentrations of 0.1 mM, while no ROS were detected in rhododendrol-treated melanocytes even at concentrations confirmed to induce cytotoxicity at 24 h (Figure 6).

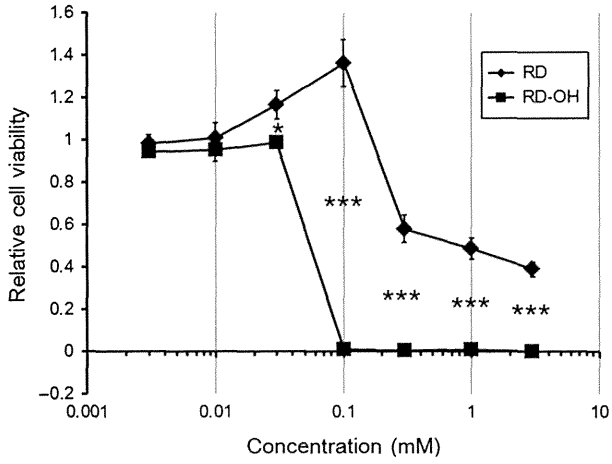

Figure 5. Hydroxyrhododendrol is more toxic in melanocytes than rhododendrol. Human melanocytes were treated with various concentrations of rhododendrol (RD) or hydroxyrhododendrol (RD-OH) for 24 h, and the cell viability was then evaluated by alamarBlue assay (*P < 0.05, ***P < 0.001, Student's *t*-test). Results are expressed as mean ± SD of triplicate experiments.

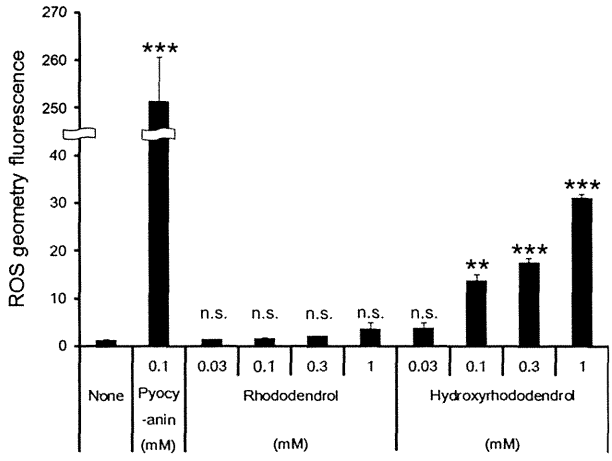

Figure 6. Hydroxyrhododendrol but not rhododendrol induces cellular ROS. The melanocytes were treated for 3 h in rhododendrol (RD) or hydroxyrhododendrol (RD-OH). The treated cells were stained with a Total ROS/Superoxide Detection kit and analyzed by flow cytometry. Pyocyanin-treated melanocytes were prepared as positive controls. Ten thousand cells were analyzed in each treatment group. The levels of ROS geometry fluorescence are shown as mean fluorescence intensities (mean ± SD). Each experiment was performed in triplicate (**P < 0.01, ***P < 0.001 vs. non-treated, Tukey's test).

Rhododendrol activates a tyrosinase-dependent ER stress response

We investigated whether rhododendrol induces ER stress and UPR followed by up-regulation of IL-8. The gene expression of CCAAT-enhancer-binding protein homologous protein (CHOP), a transcription factor with a major role in UPR-induced cell death, was found to be up-regulated in melanocytes exposed to rhododendrol for 6 h at concentrations higher than 0.3 mM. This up-regulation was clearly abolished in melanocytes treated with tyrosinase siRNA (Figure 7A). In a parallel experiment, however, the same treatment with tyrosinase siRNA did not abolish the up-regulation of CHOP by thapsigargin, an inhibitor of sarco/ER calcium ATPase and a well-known inducer of the UPR. IL-8 release was found to be up-regulated in melanocytes exposed to rhododendrol for 24 h at concentrations higher than 0.3 mM. This up-regulation was clearly abolished in melanocytes treated with tyrosinase siRNA (Figure 7B), as seen with

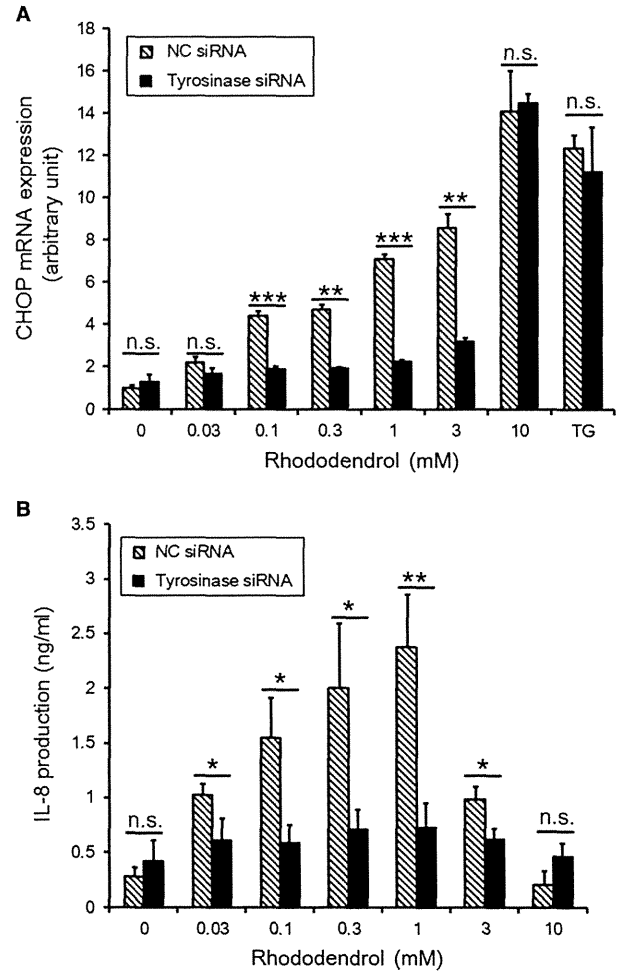

Figure 7. Rhododendrol induces CHOP mRNA expression and IL-8 production in a tyrosinase-dependent manner. Melanocytes were treated for 6 and 24 h with rhododendrol at graded concentrations, together with tyrosinase or negative control (NC) siRNA. (A) The gene expression of CHOP in melanocytes treated with rhododendrol for 6 h was detected by real-time PCR. Thapsigargin (TG) was used as a positive control inducing ER stress. (B) The IL-8 was detected using an ELISA system in the melanocyte cultured media treated with rhododendrol for 24 h. Results are expressed as mean ± SD of triplicate experiments (*P < 0.05, **P < 0.01, ***P < 0.001, Student's *t*-test).

CHOP mRNA. Thus, rhododendrol induces a tyrosinase-dependent ER stress response.

Rhododendrol induces caspase-3 activation in human melanocytes, and the tyrosinase siRNA inhibits that activation

To confirm whether the tyrosinase-dependent cell death induced by rhododendrol is apoptotic, we treated melanocytes with rhododendrol at concentrations of 0.1 and 1.0 mM for 24 h with tyrosinase or negative control siRNA. Rhododendrol increased the cleaved caspase-3 signal band by a significant amount (about 17 kD), whereas tyrosinase siRNA treatment suppressed the caspase-3 activation to the level detected in untreated control cells (Figure 8). Rhododendrol-induced caspase-3 activation is tyrosinase dependent.

Discussion

In this report, we examined the possible mechanisms of rhododendrol, a compound to cause a skin depigmentation principally at the site of repeated chemical contact. When tyrosine hydroxylase activity was measured with L-tyrosine as a substrate, rhododendrol competitively inhibited mushroom tyrosinase with a Ki value of 24 μM (Figure S2). An IC_{50} value of 5.3 μM was obtained using human melanocytes as the enzyme source, indicating that rhododendrol inhibits human tyrosinase. In fact, rhododendrol suppressed cellular tyrosinase activity in cultured human melanocytes as well as melanin synthesis in mouse B16 melanoma cells (Figure S1). On the other hand, rhododendrol was found to act as a substrate for tyrosinase when the activity was measured using [^{3}H]-rhododendrol as a substrate. The apparent Km values for rhododendrol and L-tyrosine were 0.27 mM and 0.36 mM, respectively, suggesting that rhododendrol has an affinity to the enzyme equivalent to that of L-tyrosine. These findings indicate that rhododendrol is oxidized to rhododendrol quinones by tyrosinase activity in the melanocytes, thus conferring potentially cytotoxic effects against the melanocytes. In fact, rhododendrol

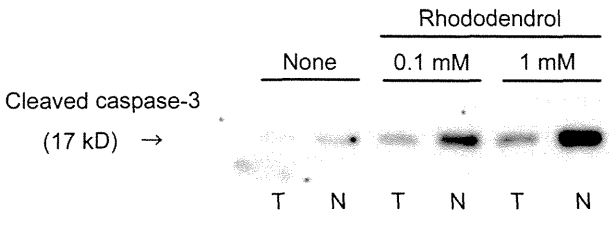

Figure 8. Rhododendrol increases cleaved caspase-3 production in human melanocytes in a tyrosinase-dependent fashion. Melanocytes were treated for 24 h with rhododendrol at concentrations of 0.1 and 1.0 mM, together with tyrosinase (T) or negative control (N) siRNA. Extracts of the treated cells were separated using SDS-PAGE and then analyzed by immunoblotting using anti-cleaved caspase-3 antibodies.

was confirmed to be cytotoxic to the melanocytes when the concentration rose to a level high enough above 0.3 mM to inhibit cellular tyrosinase. Hence, rhododendrol primarily inhibits cellular melanogenesis, but is secondarily toxic at higher concentrations when tyrosinase becomes sufficiently active to undergo rhododendrol quinone formation. In independent experiments, the IC_{50} value of cell viability with rhododendrol varied from 0.17 to 0.8 mM (Figures 4B,D, and 5). This variability in rhododendrol concentration may have been due to differences in the tyrosinase activity of melanocytes used in the experiments. We found that well-controlled melanocyte culture conditions were required for reproducible rhododendrol cytotoxicity, as higher cell density enhanced cellular tyrosinase activity (Figure S4). It also may support the notion that the cytotoxic action of rhododendrol is dependent on tyrosinase activity. For reasons we were unable to infer, a low rhododendrol concentration from 0.03 to 0.1 mM reproducibly stimulated melanocyte growth by 10% or more in repeated independent experiments (Figures 4B,D and 5).

The cytotoxicity of rhododendrol was dose dependently abolished by PTU, a chelating agent of the copper ions at the tyrosinase-active site (Klabunde et al., 1998; Olivares et al., 2002; Ryazanova et al., 2012) and a specific inducer of post-Golgi tyrosinase degradation at a concentration that inhibited rhododendrol hydroxylase activity (Hall and Orlow, 2005). A specific knockdown of tyrosinase by siRNA also overcame the cytotoxicity. These results, in sum, support the notion that the rhododendrol effect is specifically dependent on the tyrosinase essential for rhododendrol hydroxylase activity. The rhododendrol at the 10 mM concentration conferred cytotoxic action independent of tyrosinase (Figure 4D), inducing a nonspecific cell death of a type generally seen with high doses of chemicals.

The phenolic compound 4-TBP conferred cytotoxic action independent of tyrosinase activity in experiments by Yang and Boissy (1999), even though it is a tyrosine analog that binds to the catalytic site of the tyrosinase enzyme and acts as a competitive inhibitor of tyrosinase. In further studies, the melanocyte toxicity of 4-TBP was found to exert a melanocyte toxicity closely correlated with the level of TRP-1, and ultimately to induce melanocyte apoptosis (Yang et al., 2000). Our group managed to clearly prove the tyrosinase dependency of the rhododendrol cytotoxicity, but we were unable to rule out the involvement of TRP-1 in the toxicity, as tyrosinase and TRP-1 bind with each other to form a melanogenic protein complex that affects the stability of the respective proteins (Toyofuku et al., 2001).

The cytotoxicity of rhododendrol could have resulted from ROS derived from rhododendrol *o*-quinones and/or hydroxyrhododendrol (Figure 1). In fact, a low concentration of hydroxyrhododendrol (0.1 mM) exhibited both cytotoxicity and ROS-forming potential in cultured human melanocytes. Yet unexpectedly, no ROS were detected

in rhododendrol-treated melanocytes at concentrations high enough to confer cytotoxicity under conditions where significant ROS were detected by the addition of hydroxyrhododendrol. Even if ROS were formed from rhododendrol through tyrosinase activity, those released may become localized in the compartment of melanosomes and/or scavenged by L-cysteine, resulting in undetectable levels. Thus, toxicity conferred by external addition of hydroxyrhododendrol seems to be due to ROS, though most may be caused by rhododendrol *o*-quinones formed from hydroxyrhododendrol as a catechol in an non-enzymatic manner, as previously reported (Basma et al., 1995; Clement et al., 2002). Consequently, ROS might not be significantly involved in the cytotoxicity of rhododendrol. MBEH and 4-TBP were both reported to cause oxidative stress in melanogenic cells (Manga et al., 2006; Van Den Boorn et al., 2011). Yet MBEH-induced ROS exerted no toxicity against pigmented cells and there is no evidence to suggest that 4-TBP induces ROS-dependent cytotoxicity. As a result, the contribution of undetectable ROS to cytotoxicity remains controversial.

In other experiments, rhododendrol at concentrations higher than 0.3 mM was found to up-regulate the CHOP gene expression responsible for apoptosis and/or autophagy in the UPR system. This up-regulation was completely suppressed by tyrosinase siRNA. Furthermore, IL-8, which is produced downstream of Inositol-requiring enzyme-1 (IRE-1) in the UPR system (Toosi et al., 2012), was increased by rhododendrol and abolished by siRNA. These findings prove that rhododendrol-induced ER stress took place via the tyrosinase-dependent oxidation of rhododendrol. We also detected the activation of caspase-3 protein, an apoptotic-related protein, in the rhododendrol-treated cells, and found that they, too, were reduced by siRNA. The activation of caspase-3 implicates apoptosis following the induction of ER stress as a driver of cytotoxicity, though other pathways independent of ER stress might also play a role.

The cytotoxicity of rhododendrol was apoptotic and tyrosinase dependent, even though ROS were not detected at significant levels. It remains obscure how the tyrosinase-catalyzed oxidation of rhododendrol leads to ER stress followed by apoptotic cell death. Recent studies have revealed that MBEH and 4-TBP both activate the UPR system, and elicit up-regulation of IL-6 and IL-8, while both also induce autophagic melanosome degradation (Toosi et al., 2012; Van Den Boorn et al., 2011). On the other hand, the cell death in response to MBEH is necrotic, whereas that in response to 4-TBP is apoptotic (Hariharan et al., 2010; Yang et al., 2000). Hence, the activation of ER stress may sometimes lead to modes of cell death other than apoptosis. The cytotoxicity of MBEH and 4-TBP turned out not to correlate with the tyrosinase or cellular pigment synthesis (Hariharan et al., 2010; Van Den Boorn et al., 2011; Yang et al., 2000), though the ROS production was found to be dependent on the melanogenicity. Raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) has been reported to cause occupational leukoderma, and to inhibit the mouse B16 melanoma and HT1080 fibrosarcoma cell growth (Fukuda et al., 1998a,b). Although the mechanisms behind these actions and the reason for tyrosinase dependency remain obscure, raspberry ketone functions as a substrate of mushroom tyrosinase and activates melanoma tyrosinase without inhibiting melanogenesis. These findings suggest that phenolic compounds do not always induce the melanocyte cytotoxicity by the same chain of events; namely, the initial conversion of phenols to active products by tyrosinase in the melanosome, the accumulation of oxidative stress, the activation of the UPR system, and the induction of apoptotic and/or necrotic cell death. As proposed by Van Den Boorn et al. (2011) and Toosi et al. (2012), ER stress followed by IL-6 and IL-8 production may be important from an immunopathological perspective, and may enhance the melanocyte-specific antigen presentation by activating the proteasome- and/or autophagy-dependent degradation of the unfolded proteins possibly produced by ROS or active quinones.

In conclusion, our findings reveal a melanocyte cytotoxicity that may be triggered by the tyrosinase-dependent conversion of rhododendrol to active product(s). The tyrosinase-dependent accumulation of ER stress and/or activation of the apoptotic pathway may be involved in this melanocyte cytotoxicity. These findings could be helpful for the clinical diagnosis and treatment of the rhododendrol-induced depigmentation of the skin, as well as our understanding of skin depigmentation caused by similar *p*-alkylphenols. Further studies to clarify the relationship between the cytotoxicity and molecular events following the rhododendrol-tyrosinase interaction are awaited. Immunological and/or genetic studies would also be helpful for clarifying individual susceptibility to the rhododendrol-induced depigmentation of the skin.

Methods

Reagents

Rhododendrol (4-(4-hydroxyphenyl)-2-butanol)) was prepared in racemic form by reducing raspberry ketone (4-(4-hydroxyphenyl)-2-butanone) with Raney Ni in EtOH (Carruthers, 1978). Hydroxyrhododendrol was prepared from 4-(3,4-dihydroxyphenyl)-3-buten-2-one by the same procedure. 4-(3,4-dihydroxyphenyl)-3-buten-2-one was synthesized from 3,4-dihydroxybenzaldehyde and acetone, following a procedure similar to that described by Gettler and Hammett (1943). Radiolabeled rhododendrol (3′, 5′-[^{3}H]-rhododendrol) with tritium substitution at the 3- and 5- positions of hydrogen in the benzene ring was synthesized by a conventional method. It means that the unlabeled molecule is first brominated and then tritiated. The structure of most of the resulting compound was confirmed by ^{3}H-NMR spectra showing singlet signal and HPLC-co-elution with an authentic cold standard. Radiolabeled tyrosine (3′, 5′-[^{3}H]-tyrosine) was purchased from American Radiolabeled Chemicals Inc. (Saint Louis, MO, USA). PTU was purchased from Sigma-Aldrich (Saint Louis, MO, USA). Thapsigargin and L-tyrosine were purchased from Wako Chemical (Osaka, Japan). Pyocyanin was purchased from Cayman Chemical (San Diego, CA, USA).

Cell culture

Normal human epidermal melanocytes were purchased from Kurabo (Osaka, Japan). Cells were suspended (1 or 5 × 10^5 cells/ml) in MCDB153 medium supplemented with 1% Human Melanocyte Growth Supplement (HMGS; Life Technologies, Carlsbad, CA, USA), and seeded in culture plates of various sizes appropriate for the ensuing experiments. In the RNAi experiment, melanocytes were suspended (1.5 × 10^5 cells/ml) in MCDB153 medium supplemented with 0.4% bovine pituitary extract (Kurabo), 1 ng/ml recombinant basic FGF (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 500 ng/ml hydrocortisone (Sigma-Aldrich), 10 ng/ml PMA (Wako Chemical), and 0.1 mM $CaCl_2$ (Wako Chemical).

Determination of protein synthesis

Protein synthesis was evaluated by measuring the incorporation of [^{3}H]-leucine (GE Healthcare, Little Chalfont). Normal human melanocytes were seeded into 12-well culture plates at a density of 1 × 10^5 cells per well and allowed to attach overnight. Twenty-four hours after switching to another medium containing rhododendrol, the cells were incubated with 1 μCi of [^{3}H]-leucine per well for an additional 24 h. The cells were then washed twice with PBS, treated with 10% TCA at 4°C for 30 min, and solubilized in 1 N NaOH at 60°C for 1 h. The radioactivity of the solution was determined in a liquid scintillation counter (LSC1000, Hitachi Aloka Medical, Tokyo, Japan).

Cell viability assay

To study cytotoxicity, 2.5 × 10^5 cells were seeded onto 24-well plates in MCDB153 medium supplemented with 1% HMGS. Two days after plating, the cells were treated with various concentrations of rhododendrol and hydroxyrhododendrol for 24 h, and the cell viability was evaluated by alamarBlue assay (Life Technologies). When necessary, PTU treatment was started 24 h before the rhododendrol treatment.

Tyrosine/rhododendrol hydroxylase assay in cultured melanocytes

The tyrosine or rhododendrol hydroxylase activity in cultured melanocytes was determined according to the method of Oikawa et al. (1972). Normal human melanocytes were seeded in 12- or 24-well plates and cultured in MCDB153 medium supplemented with 1% HMGS at a density of 1 × 10^5 or 2.5 × 10^5 cells per well, and allowed to attach overnight. After switching to another medium containing rhododendrol or PTU, the cells were incubated for 24 h with 1 μCi of [^{3}H]-tyrosine or [^{3}H]-rhododendrol per well. In the assay of rhododendrol hydroxylase activity, the concentrations of unlabeled tyrosine and rhododendrol in culture media were equalized. A 500 or 750 μl of 10% TCA containing 20% charcoal (charcoal solution) was then added to an equal volume of medium, and the mixture was mixed in a vortex for 30 s and centrifuged at 10 000 rpm for 10 min. A 750 μl of the supernatant was then transferred to a new tube and treated twice with the charcoal solution. The radioactivity of the tritiated water produced in the final supernatant was determined in a liquid scintillation counter.

Tyrosine/rhododendrol hydroxylase assay using mushroom tyrosinase

Tyrosine or rhododendrol hydroxylase activity was determined according to the method of Pomerantz (1966). Twenty or 10 units of mushroom tyrosinase (Sigma-Aldrich) were incubated in 500 μl of PBS (pH 7.2) containing 0.1 or 1.0 mM DOPA and various concentrations of tyrosine or rhododendrol at 37°C. Radiolabeled substrate was added to the reaction mixture at a constant proportion of each cold substrate at various concentrations. After ceasing the reaction by adding 500 μl of charcoal solution, the radioactivity was measured in supernatant obtained by the same procedures described above.

Kinetic analysis of mushroom tyrosinase

Enzyme kinetics were studied by the Lineweaver–Burk plot method. Various concentrations of substrate with or without rhododendrol were added to a reaction mixture. Measured values obtained from an in vitro study were used to plot the 1/velocity against the 1/substrate concentration. Straight lines obtained by the least squares method were used to determine Km values and the type of inhibition.

Transfection

A day after the plating, the cells were transiently transfected with 10 nM of Stealth RNAi™ siRNA, including three different sequences targeting tyrosinase mRNA (Life Technologies) or negative control siRNA (Life Technologies) using Lipofectamine RNAiMAX (Life Technologies). The effects of the treatment on the expression levels of mRNA and protein were confirmed at 48 h after transfection. The analytical method is outlined in detail below. Four days after transfection, the cells were treated with various concentrations of rhododendrol for 24 h.

Gene expression assay

Total RNA was extracted using an RNeasy kit (QIAGEN, Düsseldorf, Germany). Reverse transcription was carried out using a high-capacity cDNA Archive kit (Life Technologies). Real-time PCR was performed on the StepOne Real-Time PCR system (Life Technologies) using TaqMan Universal PCR Master Mix and TaqMan Gene expression probes (Life Technologies) for genes of interest according to the manufacturer's instructions. The amount of mRNA was calculated from the cycle threshold value, that is, the experimentally determined number of PCR cycles required to achieve threshold fluorescence. The levels of gene expression were standardized with those of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA.

Analysis of ROS generation by flow cytometry

ROS generation was measured by flow cytometry with a Total ROS/Superoxide Detection kit (ENZ-51010: Enzo Life Sciences Inc., Farmingdale, NY, USA). Briefly, 3.6 × 10^4 cells were harvested, stimulated by rhododendrol or hydroxyrhododendrol at concentrations of 0.03, 0.1, 0.3, and 1.0 mM, and loaded with ROS/Superoxide Detection Mix in a MCDB153 medium containing 1% HMGS in the dark for 3 h at 37°C under a 5% CO_2 atmosphere. Fluorescence intensity was quantified using a FACSCalibur ™ flow cytometer (BD Biosciences, San Jose, CA, USA) at the FL1 channel within 1 h. A minimum of 9000 cells were analyzed in each treatment group, and the ROS-induced cells were analyzed using CellQuest software (BD Biosciences).

SDS-PAGE and immunoblotting

The cells were rinsed twice with HEPES buffer and then incubated for 1 min in lysis buffer (2% sodium dodecyl sulfate, 100 mM Tris-HCl (pH 6.8), Complete™ protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany), and PhosSTOP phosphatase inhibitor cocktail (Roche Applied Science)). The cells were then scraped, sonicated, and centrifuged at 14 000 × *g* for 10 min at 4°C. Supernatant was collected, and the protein concentrations in the lysates were quantified using a DC Protein Assay kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. Extracts were separated by SDS-polyacrylamide gel electrophoresis SDS-PAGE in NuPAGE 4–12% Bis–Tris Gels (Life Technologies) according to the manufacturer's instructions. After electrophoresis,

the protein extracts were transferred onto a polyvinylidene difluoride (PVDF) membrane using Xcell Sure Lock Mini-Cell with Xcell II Blot Module (Life Technologies).

The protein-transferred PVDF membranes were incubated for 1 h in 5% skim-milk containing buffer and then reacted with a monoclonal mouse anti-Tyrosinase antibody (T311, Sigma-Aldrich,; dilution in 1:30,000) or a monoclonal rabbit anti-Cleaved Caspase-3 antibody (Asp175) (5A1E) (#9664, Cell Signaling, Danvers, MA, USA; dilution in 1:10 000). After washing, the membranes were incubated for 1 h with peroxidase-conjugated goat anti-mouse Ig (1858413, Life Technologies; dilution in 1:2000) or peroxidase-conjugated goat anti-Rabbit Ig (P0448, Dako, Glostrup, Denmark; dilution in 1:4000). The target bands were visualized using an enhanced chemiluminescence kit (Supersignal West Dura Chemiluminescent Substrate, Life Technologies).

Detection of IL-8 by enzyme-linked immunosorbent assay (ELISA)

IL-8 was detected in the culture medium of human melanocytes. Equal numbers of cells (1.25×10^6 cells/well) were cultured in 6-well plates filled with 2.5 ml of media. The culture media was subsequently collected, briefly centrifuged, and concentrated using filtered centrifuge columns. The concentration of IL-8 in the equal volume of concentrated media was determined by ELISA [Quantikine® ELISA, Human CXCL8/IL-8 (D8000C, R&D Systems, Minneapolis, MN, USA)] according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± SD. Statistical significance was assessed by Tukey's test or Student's *t*-test using EXSUS Ver. 8.0.0 (CAC EXICARE Corporation, Tokyo, Japan). $P < 0.05$ was considered to indicate statistical significance.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Rhododendrol decreases melanin content in B16 melanoma cells without cytotoxicity.

Figure S2. Determination of the Ki value by a Dixon plot.

Figure S3. Rhododendrol exerted cytotoxicity in several human melanocyte strains and murine B16 melanoma cells via a tyrosinase-dependent mechanism.

Figure S4. The tyrosine hydroxylase activity correlated with the seeding density of the human melanocytes.

臨床トピックス

化粧品による皮膚障害

松　永　佳世子*

はじめに

いま，2つの化粧品・医薬部外品の健康被害が社会的に大きな問題になっている．その一つは旧茶のしずく石鹸に含まれた加水分解コムギ末の経皮・経粘膜感作即時型コムギアレルギーであり，他の一つはロドデノール誘発性脱色素斑（白斑）である．筆者は学会（前者は日本アレルギー学会，後者は日本皮膚科学会）の特別委員会委員長として実態の疫学調査，原因調査発生機序の解明，および患者と医師への正しい情報の提供などを行っている．

本稿においては現在の医療現場から見た化粧品の安全性の現状を紹介し，より安全な化粧品を供給し使用するための医師の症例情報を中心とした産学官連携システムを提案する．

Ⅰ．化粧品・医薬部外品とは

化粧品とは薬事法第2条第3項で「人の身体を清潔にし，美化し，魅力を増し，容貌を変え，又は皮膚若しくは毛髪を健やかに保つために，身体に塗擦，散布その他これらに類似する方法で使用されることが目的とされている物で，人体に対する作用が緩和なもの」と定義されている．医薬部外品は同第2条第2項で定義されているが，医薬部外品にはヘアダイ，パーマネント剤などが含まれ，私たちの生活の質（quality of life：QOL）を向上させ，活力を増すことができる．

Ⅱ．化粧品による皮膚障害の分類

化粧品による皮膚障害としては1）刺激性接触皮膚炎，2）アレルギー性接触皮膚炎，3）光毒性接触皮膚炎，4）光アレルギー性接触皮膚炎，5）非アレルギー性接触蕁麻疹，6）アレルギー性接触蕁麻疹，7）痤瘡の増悪などがあげられる．これに，1）および2）の結果として，色素沈着が顕著となる色素沈着性接触皮膚炎があり，黒皮症はこれにあたる．接触皮膚炎診療ガイドラインに詳細が記載されているので，参考にしていただきたい[1]（http://www.jsdacd.org/html/contact_dermatitis_guideline.pdf.）．

—Key words—
化粧品，皮膚障害，加水分解コムギ，アナフィラキシー，ロドデノール

＊Kayoko Matsunaga：藤田保健衛生大学医学部皮膚科学

Ⅲ．加水分解コムギグルパール19S®（GP19S）によるコムギアレルギー

近年，旧茶のしずく石鹸®に含まれた加水分解コムギ末GP19Sが原因で即時型コムギアレルギー症例が多発し，社会問題となっている[2~6]．2014年3月現在で2,152例の多数の確実例が登録されており，その96％が女性で平均年齢は45.8歳であった．コムギ摂食時の症状は25％がショック，30％が呼吸困難・消化器症状を伴い全体で55％がアナフィラキシー等で生命の危機を脅かされた重症例であった．

GP19Sを0.3％含む旧茶のしずく石鹸®は泡立ちのよさと美白効果があるとの口コミ，およびテレビでの宣伝などから2004年3月から2010年9月までに4,667,000名に合計46,508,000個が販売されたヒット商品であった．洗顔石鹸という用途に使用したため，皮膚および眼や鼻の粘膜を通して長期に界面活性剤とともにGP19Sに曝露された．コムギ摂食時に惹起される最初のアレルギー症状が眼瞼浮腫であったことは眼周囲の抗原曝露と吸収が多かったことを示唆する．GP19Sはグルテンを塩酸と高温で1時間程度処理し加水分解されたコムギで，蛋白質は平均分子量約50kDa，幅広いスメア状で．グルタミンがグルタミン酸に転換されたγ-グリアジンや低分子グルテニンなどを多く含み，これが原因抗原と考えられている．

Ⅳ．ロドデノール誘発性脱色素斑

「医薬部外品有効成分“ロドデノール”4-(4-ヒドロキシフェニル)-2-ブタノール」の配合された製品の使用者の中に色素脱失を生じた症例が確認され，2013年7月4日にロドデノールを含有する化粧品の自主回収が発表された．日本皮膚科学会では「ロドデノール含有化粧品の安全性に関する特別委員会（委員長　松永佳世子)」を2013年7月に発足し活動を開始した．

ロドデノール誘発性脱色素斑 Rhododenol induced-leukoderma とはロドデノール（一般名 rhododendrol）含有化粧品を使用後，主に使用部位に生じる様々な程度の脱色素斑で，使用中止により一部あるいは全体に色素再生が見られることが多い疾患である．診断基準の必須項目は1．ロドデノール含有化粧品の使用歴があり，2．ロドデノール含有化粧品を使用する前には脱色素斑がなく，使用後使用した部位におおむね一致して生じた完全ないし不完全脱色素斑がある．以上に加えて以下の小項目の一つを満たすものを各実例としている．1．使用中止により（必須項目2の）脱色素斑の拡大が使用中止後およそ1か月以内に停止した．2．使用中止により（必須項目2の）脱色素斑の少なくとも一部に色素が再生した．

ロドデノールは㈱カネボウ化粧品が独自に開発したメラニンの生成を抑える物質で，いわゆる“美白効果”を持つ物質として㈱カネボウ化粧品の製品の中で美白効果を謳った商品の多くに含まれていた．しかし，すでにほとんどの製品は自主回収されている．本例は，㈱カネボウ化粧品ではすでに18,692例（2014年3月31日現在）の被害例があり，そのうち，78.3％が回復傾向にあると報告している（http://www.kanebo-cosmetics.jp/information/correspondence/data.html#symptom_data）．日本皮膚科学会ロドデノール含有化粧品の安全性に関する特別委員会は患者向けのFAQ（http://www.dermatol.or.jp/news/news.html?id=174），および医療者向けの診療の手引き（http://www.dermatol.or.jp/info/news.html?id=108）を学会のホームページに掲載し，広報できる情報が得られた段階で改訂を繰り返してきた．現在，その発症機序については，新しい知見が集積してきており，学術誌等に投稿中である．発症要因としての個体の要因については，ゲノムの研究を開始している．

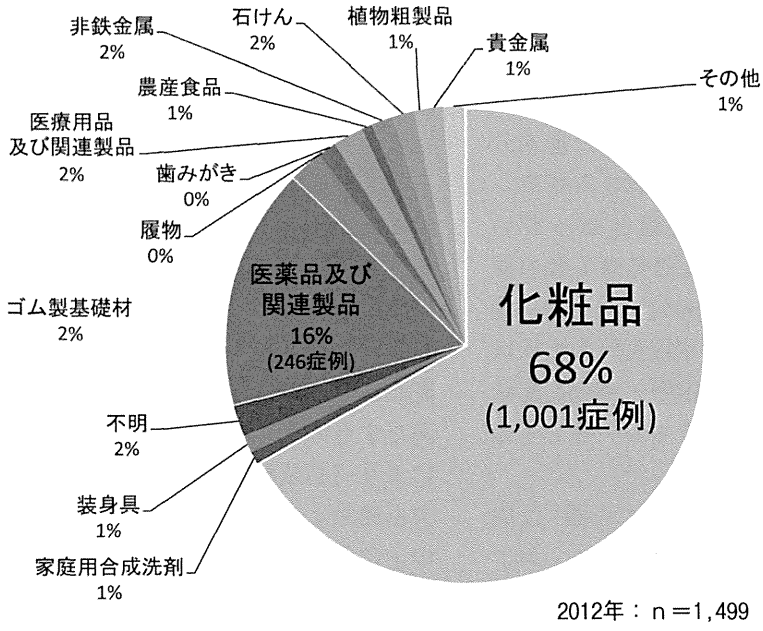

図1　原因製品種類別の全体に占める割合（2012年）
分類：総務省「日本標準商品分類」
http://www.stat.go.jp/index/seido/syouhin/2index.htm

Ⅵ. アレルギー性接触皮膚炎症例2010年から3年間の疫学調査から見えること

化粧品皮膚障害のなかで，最も確実に，また，日本全国からの疫学情報を得ているのは，アレルギー性接触皮膚炎である．日本皮膚アレルギー・接触皮膚炎学会パッチテスト試薬共同研究委員会では，2010年4月から，1年ごとのアレルギー性接触皮膚炎症例を学会員全員にアンケート調査し，その結果を集計してきた．3年間に全国の146施設の医療機関からアンケート結果を得た．原因として同定された製品を総務省日本標準商品分類（http://www.stat.go.jp/index/seido/syouhin/2index.htm）に基づいて分類した2012年度の結果を図1に示す．原因製品の最多は化粧品で68%を占めた．その原因製品を表1に示す．多い種類から，シャンプー，美容液，染毛剤，化粧水，洗顔剤であった．また，過去3年間に1製品3例以上の報告のあったものを検討した．この結果から，旧茶のしずく石鹸が自主回収した2011年5月前年の2010年に報告数が多く，その後，減少していた．また，2013年7月に自主回収したロドデノール含有化粧品も2012年度には複数の製品が報告されていた．ロドデノール誘発性脱色素斑の症例の約15%はロドデノール2%白色ワセリンに陽性反応を示している．つまり，アレルギー性接触皮膚炎が合併した症例があり，ここで，報告された症例にも脱色素斑の症例が含まれていた．これらの疫学調査が年度末の報告ではなく随時報告されるシステムをとるならば，旧茶のしずくによる即時型コムギアレルギーの発症例や，ロドデノール誘発性脱色素斑の発症をもっと早く察知し得た可能性がある．

おわりに

化粧品の安全性は1）原料の段階で，2）製