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.

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

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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.

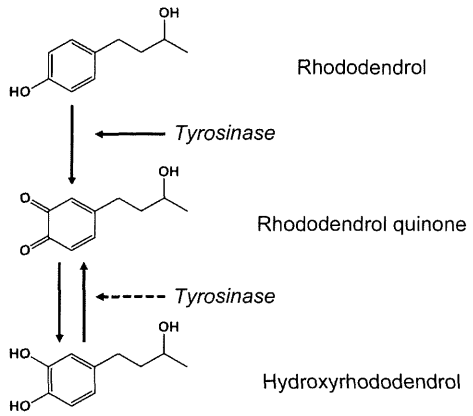

Figure 1. Outline of putative metabolic pathway involved in generation of hydroxyrhododendrol or *o*-quinone from rhododendrol.

(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).

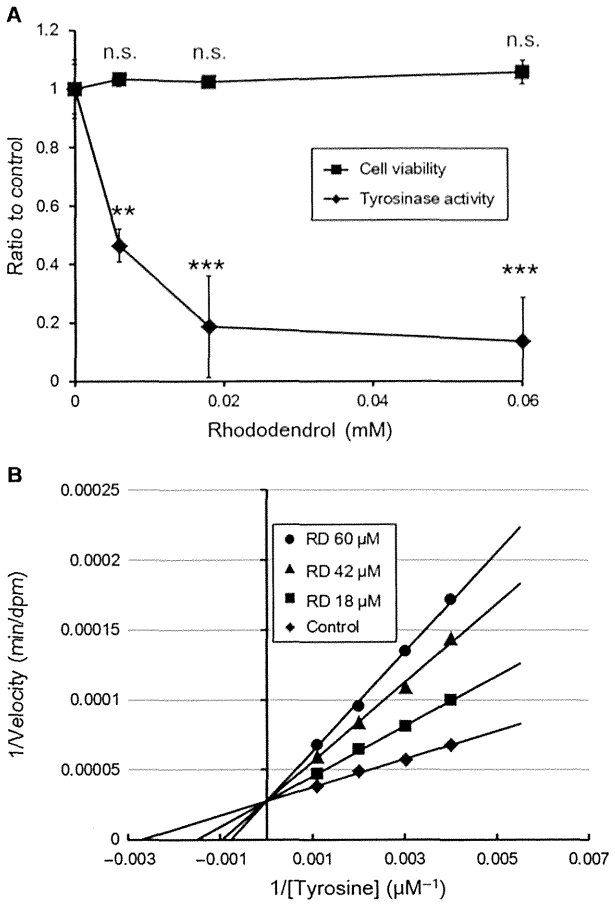

Figure 2. Rhododendrol inhibits tyrosinase when estimated with L-tyrosine as a substrate. (A) Tyrosine hydroxylase activity and cell viability estimated by protein synthesis were evaluated in cultured human melanocytes treated with various concentrations of rhododendrol. Results are expressed as mean ± 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).

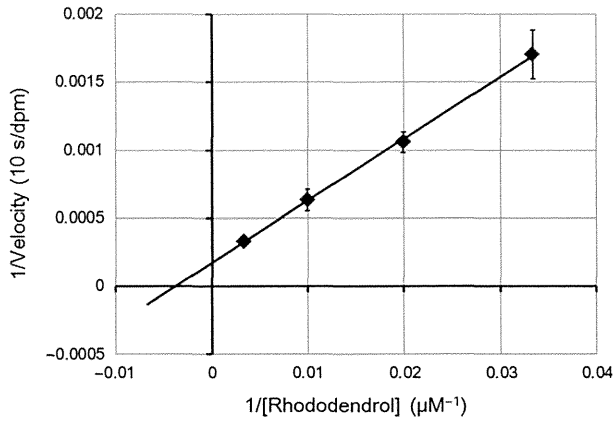

Figure 3. Lineweaver–Burk plot of tyrosinase using rhododendrol as a substrate. Ten units of mushroom tyrosinase was incubated in 500 μl of PBS containing 1.0 mM DOPA and 0.03–0.3 mM of rhododendrol with a constant proportion of radiolabeled rhododendrol at 37°C for 10 sec. The inverse of the X-intercept 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.

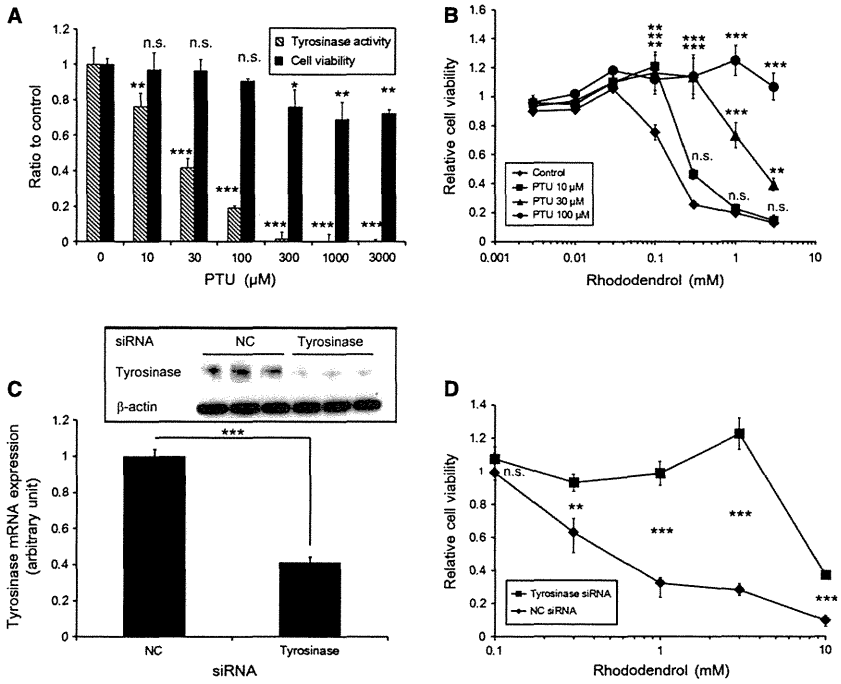

Figure 4. Tyrosinase activity is required for the melanocyte cytotoxicity induced by rhododendrol. (A) Cultured melanocytes were treated with phenylthiourea (PTU), an agent that inhibits tyrosinase via chelation of the copper ions, in order to examine the effects on rhododendrol hydroxylase activity 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).

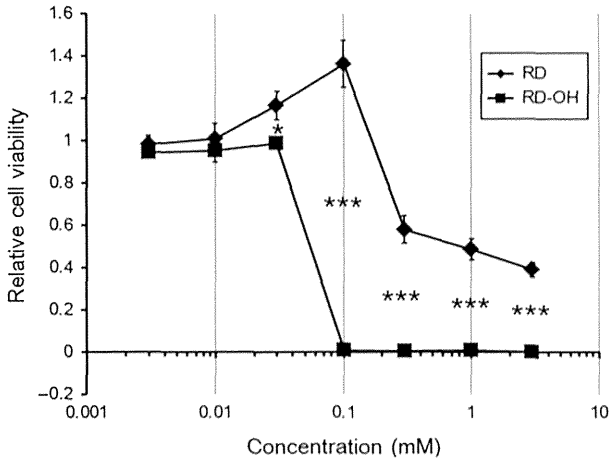

Figure 5. Hydroxyrhododendrol is more toxic in melanocytes than rhododendrol. Human melanocytes were treated with various concentrations of rhododendrol (RD) or hydroxyrhododendrol (RD-OH) for 24 h, and the cell viability was then evaluated by alamarBlue assay (*P < 0.05, ***P < 0.001, Student's *t*-test).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

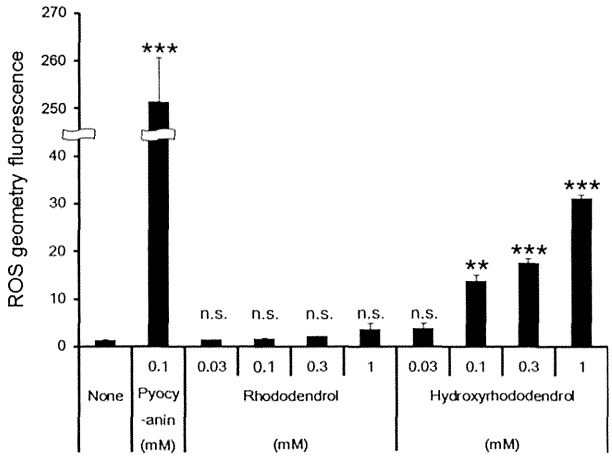

Figure 6. Hydroxyrhododendrol but not rhododendrol induces cellular ROS. The melanocytes were treated for 3 h in rhododendrol (RD) or hydroxyrhododendrol (RD-OH). The treated cells were stained with a Total ROS/Superoxide Detection kit and analyzed by flow cytometry. Pyocyanin-treated melanocytes were 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

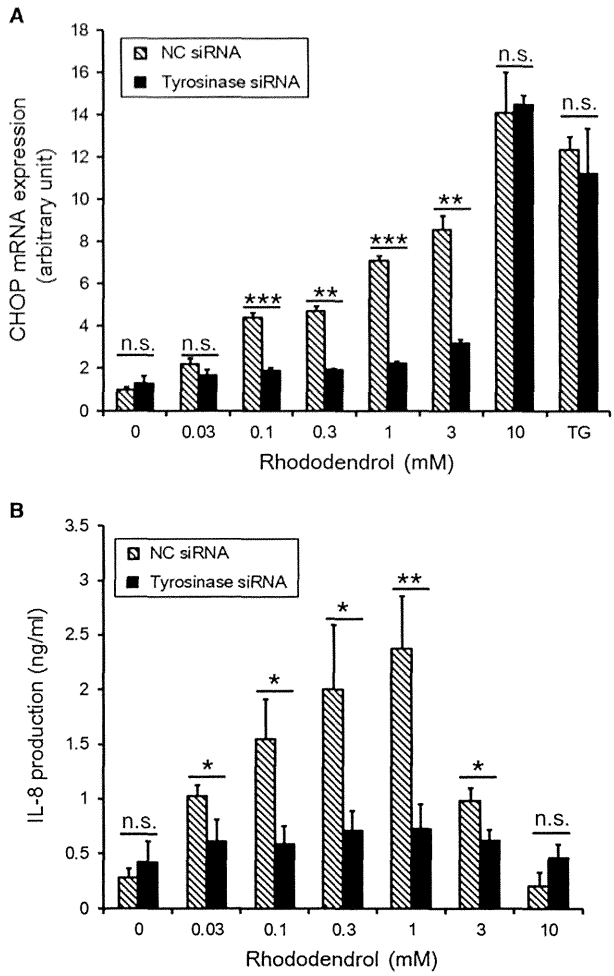

Figure 7. Rhododendrol induces CHOP mRNA expression and IL-8 production in a tyrosinase-dependent manner. Melanocytes were treated for 6 and 24 h with rhododendrol at graded concentrations, together with tyrosinase or negative control (NC) siRNA. (A) The gene expression of CHOP in melanocytes treated 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

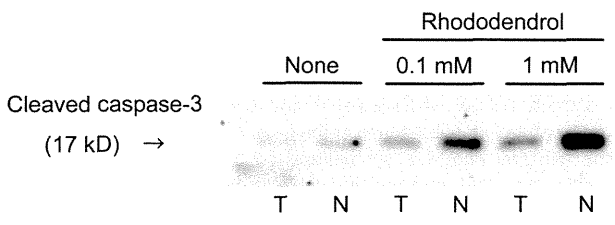

Figure 8. Rhododendrol increases cleaved caspase-3 production in human melanocytes in a tyrosinase-dependent fashion. Melanocytes were treated for 24 h with rhododendrol at concentrations of 0.1 and 1.0 mM, together with tyrosinase (T) or negative control (N) siRNA. Extracts of the treated cells were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

JAPANESE DERMATOLOGICAL ASSOCIATION
THE JOURNAL OF DERMATOLOGY

Possible allergic contact dermatitis with reticulate postinflammatory pigmentation caused by hydroquinone

Dear Editor,

Hydroquinone is one of the most prevalent skin-lightening agents. It functions by inhibiting enzymatic oxidation of tyrosine and phenol oxidases.[1] It shows sensitizing potential.[2]

A 50-year-old woman visited us with pruritic erythema with reticulate pigmentation on the face. Four months prior, pruritic erythematous macules had developed around the lip and the nasal cavity where she had used a skin-lightening product. A topical ointment containing methylprednisolone and fradiomycin sulfate had been applied. After improvement, the affected lesions had turned into hyperpigmentation. The patient had been recommended to use another skin-lightening product containing 3% of hydroquinone. The next day of application, pruritic erythema had developed. The patient had stopped using it, but the lesion turned into erythema with pigmentation. Physical examination revealed slightly pruritic erythema with reticulate pigmentation around the lip and the nasal cavity (Fig. 1a). Dermoscopy showed reticulated (arrow) and dotted (dotted arrow) hyperpigmentation and sting-like structures (bold arrow) (Fig. 1b). We did not take a biopsy specimen.

The first patch testing (International Contact Dermatitis Research Group criteria; Finn Chambers on Scanpor tape; Epitest, Tuusula, Finland) was done with the secondary used skin-lightening product as is. It showed a positive reaction to the product at D2 (+) and D4 (+).

The ingredients, provided by the manufacturer, were prepared for second patch testing as follows: the hydroquinone 3% pet., 1,2-hexanediol 1% pet., stearate/glycolate glyceryl 1% pet., royal jelly 1% pet., soybean seed extract 1% pet., polysorbate-60 5% pet., allantoin 0.5% aq. and disodium glycyrrhizinate 1% pet. Second patch testing showed a positive reaction to hydroquinone 3% pet. at D2 (+), D4 (+) (Fig. 1c) and D12 (+) (Fig. 1d), and postinflammatory reaction at D19.

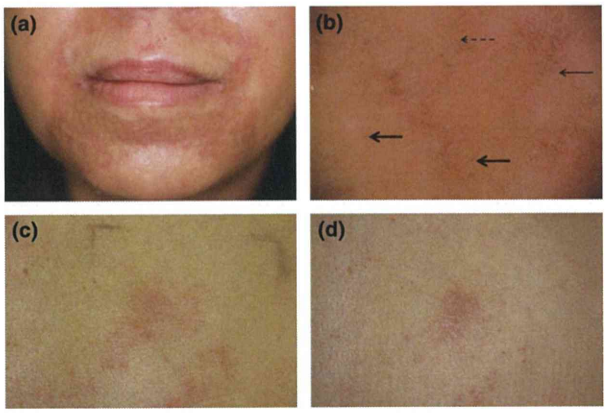

Figure 1. (a) Slightly pruritic erythema with reticulate pigmentation around the lip and the nasal cavity. (b) Dermoscopy showed reticulated (arrow) and dotted (dotted arrow) hyperpigmentation and sting-like structures (bold arrows). (c,d) A positive patch test reaction to hydroquinone 3% pet. at (c) D4 and (d) D12.

Referring to the clinical course, we surmised that delayed-type allergy to hydroquinone was sensitized during using the initially used skin-lightening product and that recurrence as allergic contact dermatitis was provoked by the secondary used one.

Hydroquinone can induce exogenous ochronosis when overused.[3] Irritant contact dermatitis can be caused dose-dependently, usually at a concentration of more than 4%.[4] In this case, we speculated that allergic contact dermatitis accelerated topical toxicity of hydroquinone itself and induced reticulate postinflammatory pigmentation. The patch tested site at D19 showed diffuse pigmentation. Ultraviolet exposure may influence reticulate pigmentation on the face.

Nath and Thappa reported the frequency of allergic contact sensitivity to hydroquinone as 4% in 25 patch-tested patients with allergic or pigmented contact dermatitis from the same bland cosmetics.[5] Allergic contact dermatitis from hydroquinone is not common. We further need to patch test with hydroquinone 0.01%, 0.1% and 1% pet. in our patient to confirm distinct allergic sensitivity, although we could not perform the third patch testing.

Exogenous ochronosis is characterized by yellow-brown, banana-shaped pigment fibers in the dermis.[3] Mishra *et al.*[3] reported multiple thin, short arciform structures as a dermoscopic figure of ochronosis. We speculated that sting-like structures in our case would be associated with ochronosis.

We need to accumulate case series of allergic contact dermatitis with particular adverse reaction caused by skin-lightening agents for the safety of customers.

CONFLICT OF INTEREST: No funding was received and no conflicts of interest declared.

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Immunoglobulin G4-related disease in a psoriasis vulgaris patient treated with ustekinumab

Dear Editor,

Immunoglobulin (Ig)G4-related disease (IgG4-RD) is a new proposed disease characterized by elevated serum IgG4 levels and the infiltration of IgG4-positive cells.[1] To our knowledge, the current case is the first one of IgG4-RD in a patient of psoriasis vulgaris treated with ustekinumab.

A 71-year-old man had a 20-year history of severe psoriasis vulgaris. Although he had been treated using a topical steroid and phototherapy, the therapeutic efficacy was limited. He was admitted to our hospital in January 2011. Although infliximab therapy was effective, it was stopped for 1 month because of the incidence of bacterial pneumonia. In March 2012, his skin condition became worse (Psoriasis Area and Severity Index [PASI], 37.9). At that time, blood examination showed the following results: white cell count, 8100/μL; C-reactive protein, 0.06 mg/dL; urea nitrogen, 16.9 mg/dL; and creatinine, 0.94 mg/dL. Whole-body computed tomography (CT) showed aorta dissection and coronary artery calcification. Thus, ustekinumab was administrated at weeks 0 and 4 and every 12 weeks (45 mg per s.c. injection).

At 14 months after initiation of ustekinumab therapy, renal function became slowly worse (serum urea nitrogen, 20.4 mg/dL; serum creatinine, 1.73 mg/dL) although his psoriatic lesion improved (PASI, 3.6). Whole-body plain CT revealed mediastinal lymphoadenopathy, retroperitoneal fibrosis and bilateral hydronephrosis (Fig. 1). Additional laboratory studies revealed the following values: white cell count, 6800/μL; C-reactive protein, 2.03 mg/dL; serum IgG, 3714 mg/dL (normal range, 800–1600); and serum IgG4, 311 mg/dL (normal, <105). These results indicated that he had IgG4-related retroperitoneal fibrosis. Although contrast enhanced CT-guided biopsy was essential for definite diagnosis, it could not be conducted because of his kidney dysfunction. Thus, he was clinically diagnosed as having IgG4-RD. We discontinued ustekinumab and started oral prednisolone therapy (1.0 mg/kg per day; 60 mg/day). Three months later, serum IgG4 levels had increased, and renal dysfunction and retroperitoneal fibrosis had largely improved. Currently, the patient takes oral prednisolone (2.5 mg/day) and cyclosporin (100 mg/day) because of recurrence of psoriasis without the exacerbation of IgG4-RD.

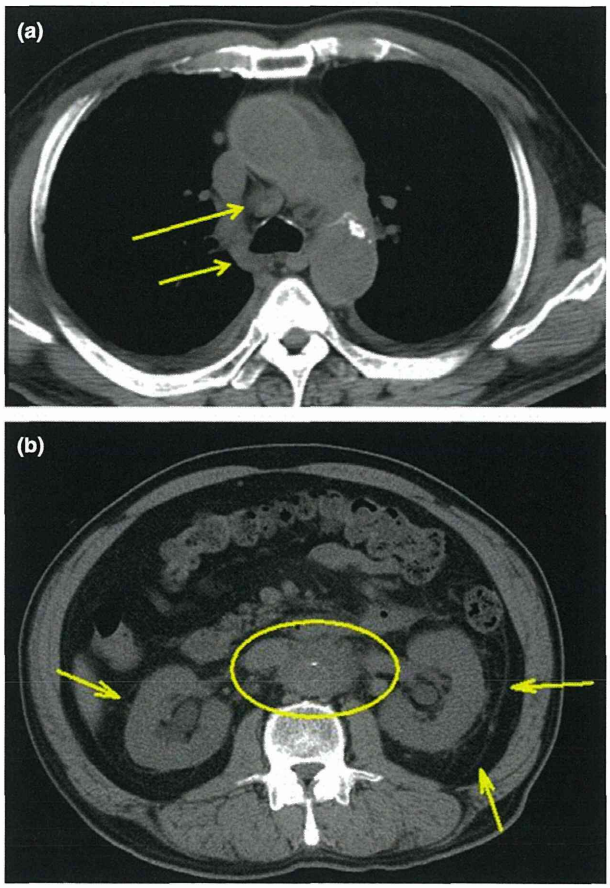

Figure 1. Computed tomography showed (a) mediastinal lymphoadenopathy (arrows) and (b) retroperitoneal fibrosis (circle) and its secondary bilateral hydronephrosis (arrows).

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ORIGINAL ARTICLE

Study of the usefulness of patch testing and use test to predict the safety of commercial topical drugs

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ABSTRACT

Patch testing (PT) can be used to identify allergens and irritants responsible for contact allergic and irritant dermatitis, respectively. However, the reproducibility of PT and correlation between PT and use test has not been fully evaluated. The aim of the present study was to examine the reproducibility of PT and its usefulness in assessing the safety of topical drugs. A total of 55 topical drugs were applied to the backs of two groups of subjects for either 24 or 48 h, and skin irritant reactions were graded at 2 and 24 h after patch removal. For the repeat open application test, six topical drugs with different irritation scores were applied to the arms of two groups of subjects twice daily for 3 weeks, and local symptoms were recorded. The mean irritation scores were similar between the two PT groups. The percentage of subjects positive for symptoms provoked by the use tests was similar between the two groups. The mean irritation scores 24 h after patch removal correlated with the skin symptoms provoked by the use test. PT was reproducible and the results correlated with the use test results. PT is a useful method for evaluating the safety of commercial topical drugs.

Key words: **mean irritation score, patch test, repeat open application test, reproducibility of results, topical drugs.**

INTRODUCTION

The ingredients contained in over-the-counter (OTC) topical drugs in Japan are regulated. Safety data regarding local irritation are not required in applications for new generic topical drugs if the ingredients conform to the standards set by the Ministry of Health, Labor and Welfare of the Japan Government.[1] Therefore, each drug manufacturer is responsible for performing safety evaluations of topical products. The type of assessment method is important in evaluating the cutaneous safety of OTC topical drugs.

The cutaneous safety of topical drugs must be tested on human subjects, and it is desirable to use the repeat open application test that reflects the clinical use of the drug. However, the test is time-consuming and may be stressful for the subjects. Therefore, patch testing (PT), a diagnostic method used to identify the cause of allergic contact dermatitis, has been used to assess the cutaneous safety of topical agents.[2]

In assessing skin irritation, skin reactions are rated and the mean skin irritation score is calculated.[2–5] Assessment of skin irritation by PT is usually performed on a limited number of subjects (~20–40) and the results may vary widely due to genetic and/or environmental differences.

Studies on evaluation of skin irritation by PT have focused mainly on variability in the visual interpretation of PT results between observers or institutions,[6–16] and comparisons between PT and *in vitro* skin irritation assays or animal data.[17–22] The reproducibility of PT reactions in the evaluation of topical drugs has not been assessed. Assessment of skin irritation by the use test has not been fully examined. Furthermore, the relationship between the results of PT and use test in testing for skin irritation remains unclear, despite reports comparing the two methods.[23–25]

In order to examine the reliability of PT in assessing the safety of topical drugs, we evaluated the reproducibility of PT and use test, and the relationship between PT and repeated open application test results in this study.

METHODS

Study design

This study was approved by the Institutional Review Board of Fujita Health University and the HUMA R&D Testing Review Board. Written informed consent was obtained from all participants. PT of 55 topical drugs commercially available in Japan was conducted on a total of 59 healthy individuals who were divided into two groups of 29 and 30 prior to testing. The skin

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reactions were rated and the mean skin irritation scores were calculated to evaluate the reproducibility between the two groups. Six of the 55 topical drugs with different skin irritation scores were applied to the arms of 52 healthy individuals for 3 weeks to simulate clinical use of the topical drugs, and the results were compared with those of PT.

PT

Subjects. A total of 59 volunteers were recruited for the PT study and were divided into two groups. Study 1 consisted of 29 subjects including four men and 25 women with an age range of 19–64 years who were living in the Aichi region in January 2011, while study 2 consisted of 30 subjects including four men and 26 women with an age range of 21–69 years who were living in either Aichi or Gifu regions in June 2011. The inclusion criteria were normal back skin and no oral or topical anti-allergic and corticosteroid drug use.

PT materials. Patch testing was conducted using 55 commercially available topical drugs, including topical antipruritic drugs, in Japan (13 creams for insect bites, 12 liquids for insect bites, 13 topical drugs for miliaria of the vulva, 17 topical drugs for dry skin) and seven control substances consisting of five skin irritants and two non-irritants (0.1% sodium lauryl sulfate [SLS] solution, 0.2% SLS, 2.0% sodium laurate solution, 0.1% benzalkonium chloride solution, 5.0% polyoxyethylene [n = 10] oleyl ether solution, distilled water, white petrolatum).[5,11,26] The concentrations of the skin irritants are likely to cause irritation.[5] The nature of the sample was not revealed until completion of the study.

PT. Using Finn Chambers on Scanpor tape (Smart Practice Japan, Yokohama, Japan), the test drugs were applied to subjects' backs and sealed for 24 (study 1) or 48 h (study 2). A total of 20 mg of ointment, cream or lotion was placed on an aluminum cup with a diameter of 8 mm. If the test drug was a liquid, a Finn Chamber filter paper disc was soaked in 15 μL of the sample. Patches had 10–12 chambers and the application site varied among individuals. The chambers were removed after 24 (study 1) or 48 h (study 2) after application, and the skin reactions at 2 and 24 h after chamber removal were assessed by a single observer blinded to the test drug. One

Table 1. Skin reaction scoring criteria

Score	Reaction
0	No reaction
1	Noticeable erythema or erythema ≤50% of the patch area
2	Minimal to moderate erythema or erythema >50% of the patch area
3	Distinct erythema
4	Erythema with papular or edematous reaction
5	Erythema with vesicular reaction
6	Corrosive reaction (bullae formation, necrosis)

Table 2. Concentrations of active ingredients (%)

Drug number	No. 1 (gel)	No. 2 (lotion)	No. 3 (liquid)	No. 4 (liquid)	No. 5 (cream)	No. 6 (liquid)
Prednisolone acetate	0.125					
Lidocaine hydrochloride	3	2	2			
Diphenhydramine		1	1 (hydrochloride)	2 (hydrochloride)	2 (hydrochloride)	2 (hydrochloride)
Urea		10	10			
Tocopheryl acetate		0.5			0.5	
Glycyrrhetinic acid		0.2		0.1	0.2	
L-menthol	3	0.5		5	0.5	
DL-camphor				1		
Others	Chlorpheniramine maleate (1)	Crotamiton (5)			Isopropylmethylphenol (0.1)	Panthenol (1)
	Benzethonium chloride (0.1)					
	Glycol salicylate (2)					

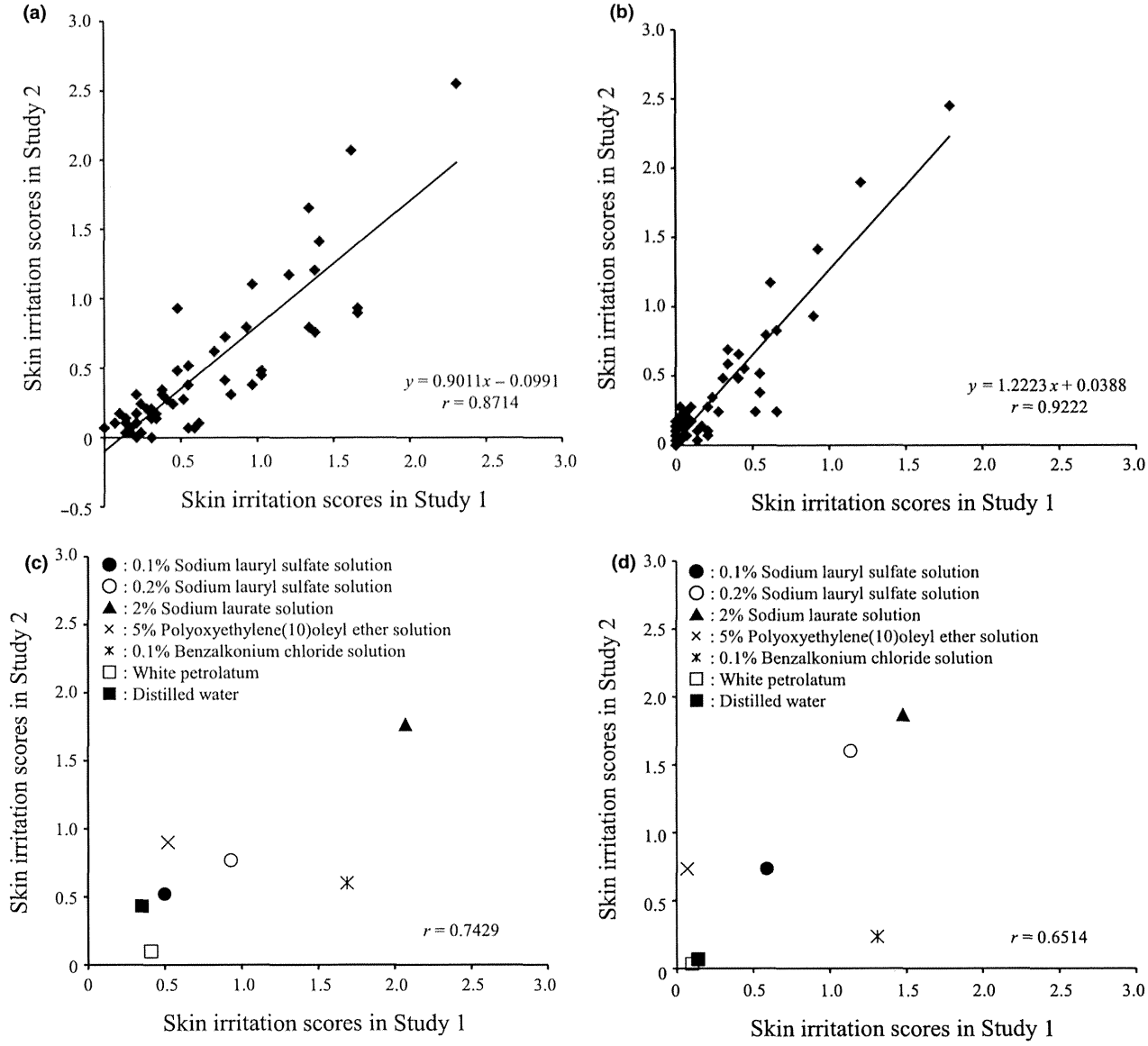

Figure 1. (a) Correlation between the mean skin irritation scores at 2 h after patch removal after 24 h (study 1) and 48 h (study 2) of drug exposure. (b) Correlation between the mean skin irritation scores at 24 h after patch removal after 24 h (study 1) and 48 h (study 2) of drug exposure. (c) Correlation between the mean skin irritation scores at 2 h after patch removal after 24 h (study 1) and 48 h (study 2) of exposure to control substances. (d) Correlation between the mean skin irritation scores at 24 h after patch removal after 24 h (study 1) and 48 h (study 2) of exposure to control substances.

dermatologist inspected the sites at 2 h after removal in study 1, and at 2 and 24 h in study 2. Another dermatologist inspected the sites at 24 h after removal in study 1. They were trained and gave ratings according to the criteria[27] for skin irritation of the skin irritation research group of the Japanese Society for Contact Dermatitis, as shown in Table 1.

Use test

Subjects. A total of 52 healthy subjects were recruited for the use test study and were divided into two groups. Study 3 consisted of 10 men and 12 women with an age range of 24–60 years who were living in the Toyama region in September 2011. Study 4 consisted of 12 men and 18 women with an age range of 21–58 years who were living in Tokyo, Kanagawa, Chiba or Saitama region in December 2013.

Test materials. Six topical drugs (Table 2) with the following mean irritation scores 24 h after patch removal were selected: 1.5 or more (n = 1); less than 0.2 (n = 1); and 0.2–1.4 (n = 4).

Table 3. Number and type of skin symptoms in study 3 and study 4

Drug No.	No. 1		No. 2		No. 3		No. 4		No. 5		No. 6	
Mean skin irritation score 24 h after patch removal (study 1/study 2)	1.79/2.45		0.34/0.59		0.52/0.24		0.62/1.17		0.66/0.83		0/0.10	
	Study 3	Study 4	Study 3	Study 4	Study 3	Study 4	Study 3	Study 4	Study 3	Study 4	Study 3	Study 4
Scaling	0	0	0	0	2	0	0	0	1	0	0	0
Erythema	6	0	1	0	0	0	2	0	1	0	1	0
Redness (transient)	4	1	12	0	12	0	8	0	9	1	7	0
Papule	1	3	2	3	0	2	0	0	0	0	0	0
Edema	0	0	0	0	0	0	0	0	0	0	0	0
Soreness	2	1	4	0	1	0	4	0	0	0	1	0
Heat	0	0	0	0	0	0	1	1	0	0	0	0
Itchiness	3	0	0	0	0	0	0	0	0	0	0	0
Miscellaneous: Skin peeling-like symptom (dry cream)	4	5	1	1	0	0	0	0	0	0	0	0
Miscellaneous: Coolness	0	0	0	3	0	0	0	0	0	0	0	0
Miscellaneous: Pigmentation	1	0	0	0	1	0	0	0	0	0	0	0
Total no. of symptoms observed	21	10	20	7	16	2	15	1	11	1	9	0
Total no. of symptoms observed excluding dried cream	17	5	19	6	16	2	15	1	11	1	9	0
Total no. of symptoms observed excluding transient redness	17	4	8	6	4	2	7	1	2	0	2	0
No. of subjects positive for symptoms Provoked by the drug (% of total 22 [study 3]/30 [study 4] subjects)	10 (45.5)	8 (26.7)	9 (40.9)	3 (10)	5 (22.7)	1 (3.3)	5 (22.7)	1 (3.3)	2 (9.1)	1 (3.3)	2 (9.1)	0
No. of subjects with objective symptoms (% of total 22 [study 3]/30 [study 4] subjects)	6 (27.3)	3 (10)	6 (27.3)	2 (6.7)	4 (18.2)	1 (3.3)	4 (18.2)	0	2 (9.1)	1 (3.3)	2 (9.1)	0
No. of subjects with subjective symptoms (% of total 22 [study 3]/30 [study 4] subjects)	4 (18.2)	5 (16.7)	4 (18.2)	2 (6.7)	1 (4.5)	0	2 (9.1)	1 (3.3)	0	0	1 (4.5)	0