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patients. In addition, CCR4+CD8+ T cells and CCL22+ not CCL17+ monocytes were preferentially found to infiltrate into the lesional skin in the affected patients, providing the possibility of their involvement in skin inflammation and hypopigmentation in RIL by chemo-attractive fashion. We believe that these results help to clarify the pathogenesis of cosmetics-induced leukoderma, which may lead to the development of novel therapeutic agents.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.02.014.

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Letter to the Editor

Immunohistopathological analysis of frizzled-4 -positive immature melanocytes from hair follicles of patients with Rhododenol-induced leukoderma

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A recent study in July 2013 reported that repeated application of racemic RS-4-(4-hydroxyphenyl)-2-butanol (rhododendrol; trade name: Rhododenol [RD]), a melanin synthesis inhibitor used in topical skin-whitening cosmetics, induced cutaneous depigmentation. Approximately 16,000 consumers developed skin depigmentation (RD-induced leukoderma) on their face, neck, and upper limbs [1]. Mechanisms underlying this condition have been investigated by performing biochemical, cytological, and immunological studies. Then, not only cytotoxic effects on melanocytes but also subsequent immune reactions have come out to be contributing to the expression of RD-induced leukoderma [2].

Repigmentation of vitiligo vulgaris frequently occurs from hair follicles, indicating the presence of inactive melanocytes that may induce repigmentation [3]. In 2002, Nishimura et al. identified melanocyte stem cells (MSCs) in the lower permanent portion of mouse hair follicles by using Dct-lacZ transgenic mice [4]. Thus, immature melanocytes such as MSCs may play a role in the repigmentation of vitiligo vulgaris. Repigmentation of RD-induced leukoderma sometimes occurs in the same manner as that of vitiligo vulgaris (Fig. 1). We histologically investigated the survival rate of immature melanocytes or MSCs in hair follicles of patients with RD-induced leukoderma. Skin biopsies were performed for patients ($n = 25$) with RD-induced leukoderma who visited Yamagata University Hospital. Two samples, i.e., from affected (depigmented) area and non-depigmented area close to the affected area (perilesion), were obtained from each patient. Informed consent was obtained from each patient, and study protocols were approved by the Ethics Committee of the Yamagata University Faculty of Medicine. Skin samples lacking hair follicles were excluded from the study. Thus, the study included 10 and 9 samples from the affecte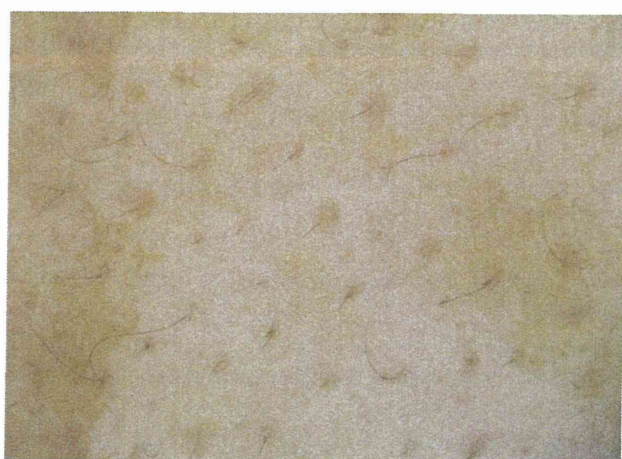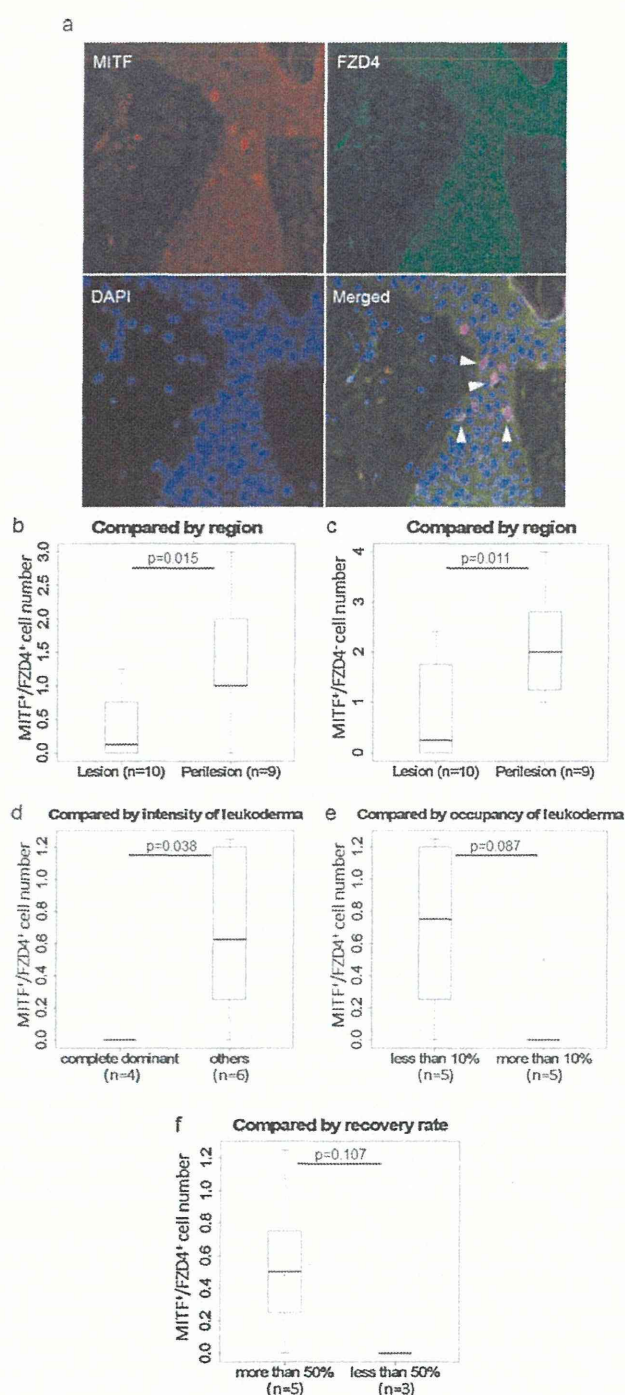d and perilesional areas, respectively. To confirm the presence of immature melanocytes or MSCs, expression of frizzled-4 (FZD4) and microphthalmia-associated transcription factor (MITF) was investigated immunohistochemically. A study in mice reported that expression of Fzd4 was higher in immature melanocytes than in melanoblasts [5], indicating that Fzd4 is a useful marker for identifying MSCs. In the present study, formalin-fixed paraffin-embedded tissue sections were deparaffinized and rehydrated. Heat-mediated antigen retrieval was performed using sodium citrate buffer (pH 6) in an autoclave (2 atmospheres, 121 °C, and 20 min) for unmasking antigens. The sections were stained with rabbit anti-FZD4 antibody (Millipore, Billerica, MA, USA) and mouse anti-MITF antibody (Thermo Fisher Scientific Inc., Fremont, CA, USA) followed by staining with appropriate secondary antibody (fluorescein-conjugated AffiniPure goat anti-rabbit antibody (H + L; Jackson ImmunoResearch Inc., West Grove, PA, USA) or rhodamine-conjugated AffiniPure donkey anti-mouse antibody (H + L; Jackson ImmunoResearch Inc.)). DAPI (Thermo Fisher Scientific Inc.) was used for nuclear staining. After observation under a fluorescence microscope, the sections were stained with hematoxylin–eosin to confirm the morphology in the same area. Number of cells in each section was counted by 2 physicians. MITF and FZD4 double-positive cells in hair follicles (Fig. 2a; perilesion from one of the patients) were counted at 400× magnification, and average cell count per section was used for statistical analysis. MITF$^+$ and FZD4 cells in hair follicles were also counted in the same manner. P-values were calculated using a two-sided Wilcoxon–Mann–Whitney test.

The following 4 observations were made. First, the number of immature melanocytes in hair follicles was significantly lower in the depigmented area than in the perilesional area ($p = 0.015$, Fig. 2b), as well as the number of MITF$^+$ and FZD4$^-$ melanocytes ($p = 0.011$, Fig. 2c). Second, immature melanocytes were not detected in patients with a complete depigmentation dominant phenotype ($p = 0.038$, Fig. 2d). Dominancy was defined as more than 60% of the total affected area, which was in accordance with that defined in a previous Japanese study [6]. Third, compared to patients with leukoderma affecting < 10% of their face and neck area, patients with leukoderma affecting larger percentage of their face and neck area had decreased number of immature melanocytes; however, the difference was not statistically significant (Fig. 2e). Finally, we compared 1-year recovery rate of the patients from their initial visit and observed that immature melanocytes were not detected in patients showing < 50% recovery rate; however, the difference was not statistically significant (Fig. 2f).

Both of the number of FZD4$^+$ and FZD4$^-$ melanocytes were significantly lower in hair follicles, suggesting that external use of RD damaged both mature and immature melanocytes such as MSCs having less or no tyrosinase activity [4,5]. While a melanocyte cytotoxicity of RD has been revealed to exert in tyrosinase-dependent manner [1], this result indicated the other mechanisms of cell damaging toward immature melanocytes such as immunological reactions as previously reported [7–9]. A correlation was observed between the number of immature cells and severity of leukoderma, indicating that the survival rate of immature melanocytes contributed to disease severity. FZD4$^+$ immature melanocytes were not detected in 3 patients showing < 50% 1-year recovery rate after their initial visit. However, these patients showed gradual improvement. It should be noted that the study included a very small number and only small sections of skin samples (3–4 mm) obtained by punch biopsy.

To our knowledge, this is the first study to examine the presence of immature melanocytes or MSCs in patients with chemical vitiligo. Our findings may help in elucidating the

Fig. 1. Dermoscopic findings of a patient with RD-induced leukoderma (4 months after discontinuing RD). Repigmentation is clearly observed in the perifollicular area.

Fig. 2. (a) Confocal microscopy of cells expressing MITF (red) and FZD4 (green). Nuclei are stained with DAPI. A few MITF and FZD4 double-positive cells (arrowheads) are observed in hair follicles (perilesion from one of the patients). (b–e) Box plot analysis of the average number of immature melanocytes per section. (b) The number of immature melanocytes was significantly lower in the depigmented area than in the perilesional area. (c) The number of MITF+ and FZD4- melanocytes was also decreased in the lesion.(d) Immature melanocytes were not detected in patients with a complete depigmentation dominant phenotype. (e) Compared to patients with leukoderma affecting <10% of their face and neck area, patients with leukoderma affecting larger percentage of their face and neck area had decreased number of immature melanocytes. (f) Immature melanocytes were not detected in patients showing <50% 1-year recovery rate after their initial visit.(For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

pathogenesis of RD-induced leukoderma and other chemical leukoderma.

Conflict of interest

The authors have no conflict of interest to declare.

Acknowledgement

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Letter to the Editor

Knockdown of either filaggrin or loricrin increases the productions of interleukin (IL)-1α, IL-8, IL-18 and granulocyte macrophage colony-stimulating factor in stratified human keratinocytes

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Filaggrin (FLG) not only provides a cytoskeleton for the cornified envelope through aggregation of keratin filaments but also makes a contribution to hydration and maintenance of acidity in stratum corneum (SC), while loricrin (LOR) composes 70% of the total protein mass of the cornified layer, which contribute to effective skin barrier [1,2]. Atopic dermatitis (AD) is a chronic dermatitis characterized by dysfunction of skin barrier and allergic inflammation. Recently, it became apparent that gene mutation of FLG is a major factor of development of human AD [1]. In addition, expressions of FLG and LOR in keratinocytes are down-regulated by inflammation. Indeed, decrease in FLG and LOR in skin of patients with AD is observed in those with or without the gene mutation [2–4]. Although FLG and LOR are theoretically involved in permeability barrier, we wondered if reduction of keratinocyte differentiation-related molecules, such as FLG and LOR, might be directly involved in cutaneous inflammation. In fact, a recent study revealed a subclinical inflammation in the patients with icthyosis vulgaris possessing loss of function mutation of FLG [5]. Hence, we examined the effects of knockdown of FLG or LOR on the production of pro-inflammatory cytokines in cultured normal human keratinocytes (NHK).

NHK transfected with siRNA for control, FLG or LOR were incubated at air-liquid interface for cell differentiation, as shown in Supplementary materials and methods, since protein levels of FLG or LOR mainly expresses in differentiated NHK [1,2]. After incubation for 7 days at air–liquid interface, we examined the mRNA in NHK and secreted proteins in media of interleukin (IL)-1α, IL-8, IL-18 and granulocyte macrophage colony-stimulating factor (GM-CSF), which are pro-inflammatory cytokines associated with inflammation on human AD as described below. After incubation for 7 days at air-liquid interface, stratification but not cornification of keratinocytes was observed under the hematoxylin and eosin staining (Fig. S1a). At that time, mRNA and proteins of FLG and LOR were suppressed in NHK transfected with siRNA for FLG or LOR, respectively, and each siRNA did not suppress another gene expression (Figure S1b, c). The mRNA expressions of IL-1α, IL-8, IL-18, and GM-CSF were up-regulated by the knockdown of either FLG or LOR. Similar results were obtained with analysis of secreted proteins in the media (Figs. 1 and 2).

The present study suggests that reductions of keratinocyte differentiation-related molecules in keratinocytes not only affect the permeability barrier functions [6] but also might directly confer a susceptibility to cutaneous inflammation via up-regulation of pro-inflammatory cytokines production, although precise mechanism for that remains unclear. The up-regulation of expression of thymic stromal lymphopoietin in FLG-knockdown keratinocytes [7] supports this idea. Of note, the up-regulations of pro-inflammatory cytokines by the reduction of FLG or LOR shown in the present study are generally consistent with the observation in human AD. Levels of IL-1α, IL-8 and IL-18 in SC of patients with AD are higher than those of healthy controls [8,9], and GM-CSF is overproduced by keratinocytes in patients with AD [10]. Notably, Kezic et al. [8] also reported that levels of IL-1α in the SC of AD patients with FLG gene mutations were significantly higher than those in AD patients without FLG gene mutation.

According to the outside to inside pathogenic mechanisms showing that permeability barrier abrogation induces production
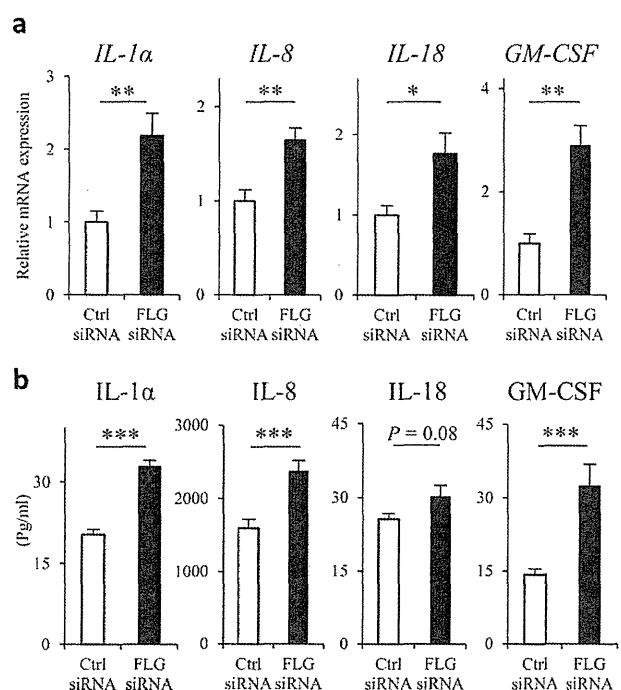
Fig. 1. Levels of mRNA (a) and secreted proteins (b) of IL-1α, IL-8, IL-18 and GM-CSF on stratified NHK transfected with siRNAs for FLG or control siRNA (Ctrl siRNA) were examined as described in the supplementary materials and methods. Levels of mRNA of IL-1α, IL-8, IL-18, and GM-CSF were up-regulated by the transfection with siRNA for FLG (a), and similar results were obtained with ELISA (b). Stratified NHK and the media were harvested 24 h after the last medium exchange, except for media for IL-8 and IL-18 which were harvested 72 h after the last medium change since no difference in levels of IL-8 and IL-18 in media were detected at 24 h (data not shown). N = 7–9 in (a) and (b). Error bars equal to means ± SEMs. *P < 0.05, **P < 0.01, ***P < 0.001.

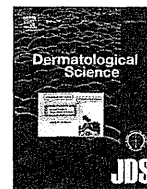Journal of Dermatological Science xxx (2015) xxx–xxx

Rhododenol-induced leukoderma in a mouse model mimicking Japanese skin

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ABSTRACT

Background: Rhododendrol, 4-(4-hydroxyphenyl)-2-butanol, Rhododenol[®] (RD), a naturally occurring phenolic compound, was developed as a tyrosinase inhibitor for skin-lightening/whitening cosmetics. In 2013, skin depigmentation was reported in consumers using RD-containing skin-brightening cosmetics; this condition is called RD-induced leukoderma.

Objective: The etiology of RD-induced leukoderma is still largely unknown. Here, to assess the depigmentation potential of RD, we developed a new mouse model of leukoderma by topically applying RD.

Methods: Hairless hk14-SCF Tg mice with melanocytes distributed in the epidermis were used for this study. RD was applied on the dorsal skin of the mice daily for 28 days. Then, immunohistological, biochemical, and electron microscopic analyses were performed on biopsy samples taken from these mice.

Results: The depigmentation in the RD-treated sites appeared on Day 14. Histological examination indicated a loss of epidermal melanocytes at Day 7. On the other hand, the melanocyte number did not decrease in the albino mice having the same background as the hairless hk14-SCF Tg, but without tyrosinase activity. Biochemical analyses showed that the eumelanin content decreased in the RD-treated sites and metabolites of RD-quinone, i.e., non-protein thiol adducts and protein-SH adducts, were produced. Electron microscopic analyses revealed double-membrane-walled structures containing electron-dense material, which might be typical for melanin-containing autophagosomes and a dilated endoplasmic reticulum (ER), which would indicate ER stress.

Conclusions: These data suggested that RD exerted tyrosinase-dependent melanocyte cytotoxicity and that tyrosinase-dependent accumulation of ER stress from activation of the autophagy pathway contributed to melanocyte cytotoxicity.

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1. Introduction

Rhododendrol, 4-(4-hydroxyphenyl)-2-butanol, Rhododenol[®](RD), a naturally occurring phenolic compound, was

Abbreviation: RD, Rhododendrol, 4-(4-hydroxyphenyl)-2-butanol, Rhododenol[®].
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developed as a tyrosinase inhibitor for skin-lightening/whitening cosmetics. In 2013, skin depigmentation was reported in consumers using RD-containing skin-brightening cosmetics; this skin condition is called RD-induced leukoderma. RD-induced leukoderma has been confirmed in about 16,000 (2%) of 800,000 estimated users of these cosmetic products and has caused broad social and customer concerns. The symptom included partial/complete depigmentation on the neck, hands, and face at the application sites of the products [1]. Yagami et al. reported that the first case of RD-induced leukoderma was accompanied by allergic contact dermatitis, and they suggest that it is necessary to consider

that the cosmetics caused not only contact dermatitis but also leukoderma [2]. A histopathological analysis of skin lesions obtained from such patients showed that the MITF and Melan-A-positive melanocyte count significantly decreased compared to that in normal skin [3].

A previous report suggested that RD-induced melanocyte toxicity was due to tyrosinase-catalyzed conversion of RD to toxic quinones and the concomitant production of reactive oxygen species [4,5]. Another study reported that no reactive oxygen species were detected in the RD-treated melanocytes, and that RD exerts tyrosinase-dependent melanocyte toxicity by concomitantly inducing endoplasmic reticulum (ER) stress and apoptosis [6]. Kasamatsu et al. [7] also reported that the melanocyte damage induced by RD was related to tyrosinase activity at a certain threshold by using 13 human melanocyte lines derived from donors of different ethnic backgrounds. Furthermore, Yang et al. [8] observed apoptotic cell death in epidermal melanocytes. Exposure of melanocytes to RD inhibited melanin production and enhanced the autophagy-lysosome pathway, leading to autophagic melanosome degradation. They found that RD-induced ubiquitin accumulation and ER stress, which is one of the causes of low viability of RD-induced melanocytes. Kuroda et al. [9] reported histological findings on repigmentation of RD-induced leukoderma in a guinea pig model. Most of these studies investigating the etiology of RD-induced leukoderma, except the report by Kuroda et al., have been carried out using in vitro systems, e.g., cultured melanocytes. It was difficult to use wild mice to investigate pigment disorders, especially acquired leukoderma, e.g., vitiligo, because melanocytes are only distributed in the ears and tail of wild mice. Therefore, we established a new mouse model with RD-induced leukoderma, by topically applying RD on the skin of hairless hk14-SCF transgenic (Tg) mice [10].

In this paper, we describe histological, morphological, and biochemical characterization of this mouse model of RD-induced leukoderma. Our findings will help clarify the etiology of similar forms of chemically induced cutaneous depigmentation, such as chemical leukoderma and idiopathic vitiligo.

2. Materials and methods

2.1. Chemicals

Rhododendrol (RD) was kindly provided by Kanebo Cosmetics Inc. (Tokyo, Japan). Ethanol (EtOH) as the vehicle was purchased from Wako Pure Chemical (Osaka, Japan). All 30% RD solutions used in this study were prepared in 50% EtOH (ethanol:water = 1:1).

2.2. Animals

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Use of Laboratory Animals and were approved by the Animal Research Committees of Yamagata University.

Hk14-SCF Tg mice were newly developed according to the method described in the previous report [10], and the background of the Tg mice was transferred to C57BL/6J. Then, the hairy hk14-SCF Tg mice were crossbred with hairless mice, Hos:HRM, also of background C57BL/6J, resulting in hairless hk14-SCF Tg mice. Hos:HRM mice were obtained from the Hoshino Laboratory Animals Inc. (Bandou-shi, Ibaraki, Japan). After generating the hairless hk14-SCF Tg mice, we chose mice having the similar color and melanocyte count as those of Japanese skin. For genotyping, tail-snip DNA was screened for SCF transgenes by PCR. These mice were maintained in pathogen-free facilities at Yamagata University, and were 10–12 week of age at the beginning of each experiment.

2.3. Assessment of melanocytes in the epidermis

Skin samples were taken from the backs of the mice. In all samples, the obtained tissues were fixed, embedded in paraffin, cut into 4 μm slices, and supplied for this study. Tissue sections were dewaxed in xylene and rehydrated through decreasing concentrations of EtOH.

For Melan-A staining, rabbit anti-Melan-A/MART-1 (NBP1-30151, 1:100, Novus biological, CO, USA) was used according to the manufacturer's instructions. The secondary antibody used in this study was Histofine Simple Stain MAX-PO (M) kit (Nichirei, Tokyo, Japan). Images were obtained using an Olympus AX80 microscope. Immunoreactivity was evaluated under light microscope and was considered specific for cytoplasmic Melan-A.

For the dopa reaction, skin biopsy samples were frozen in an optimum cutting temperature compound (Muto Pure Chemicals, Tokyo, Japan). Cryosections of samples were incubated in 0.1% DL-β-(3, 4-dihydroxyphenyl) alanine (DL-DOPA, Wako Pure Chemicals, Tokyo, Japan) in PBS at room temperature for 2 h.

2.4. Measurement of skin color

To evaluate the skin color (i.e., melanin index), the treatment area was measured by a portable spectrophotometer (Mexameter® MX18, Courage + Khazaka Electronic GmbH., cologne, Germany) every week in the hairless hk14-SCF Tg mice. Each animal was evaluated for skin color at the application site six times and an average value was calculated.

2.5. Immunofluorescence analysis

The backs of both the hairless hk14-SCF Tg mice and the albino mice were excised at 0, 7, 14, and 28 days, and at 53 days (albino mice) during the RD treatment. To observe the re-pigmentation, application of 30% RD solution to the hairless hk14-SCF Tg mice was stopped at 28 days and the mice were observed until the pigmentation returned to the baseline level. Skin specimens were taken from these mice after 42 days, from both the re-pigmentation site and the white spot site. Tissues were frozen in an optimal cutting temperature (OCT) compound. Rabbit antibody Melan-A/MART-1 (NBP1-30151) was used for melanocyte staining. Bound antibody was detected using FITC conjugated swine anti-rabbit immunoglobulins (1:20, DAKO, Carpinteria, CA, USA). Slides were mounted with slow fade (Invitrogen). Images were captured with a fluorescent microscope (Olympus BH-2). The mean number of melanocyte marker-positive cells was determined from three randomly selected fields. All cases were reviewed independently by the two authors.

2.6. Biochemical analyses

Skin samples were prepared from five hairless hk14-SCF Tg mice. The treated area was biopsied on Days 0, 3, 7, and 15. These samples were homogenized using a Ten-Broeck homogenizer in 0.4 M $HClO_4$ and fractionated into (soluble) non-protein and (insoluble) protein fractions. Biochemical analyses were performed as previously described [11]. Analyses of the soluble fraction gave values for free catechols while those of the insoluble fraction yielded values for protein-bound (PB) catechols.

2.7. Electron microscopy analyses

Skin samples of both the hairless hk14-SCF Tg mice and albino mice were biopsied on Days 0, 3, and 7. These samples were fixed with 2.5% glutaraldehyde in 2 mM cacodylate buffer at 4 °C for 60 min. The specimens were then dehydrated by passing them

through an ethanol series dilution, followed by QY-1 (*n*-butylglycidyl ethanol) before the samples were embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined under an electron microscope (Transmission Electron Microscope H-7100, Hitachi Hitechnologies, Tokyo, Japan).

2.8. Immunostaining for ER stress

To assess the induction of ER stress in melanocytes by application of RD, expression of GRP78/BiP and Melan-A/MART-1 was investigated immunohistochemically using serial sections. Skin samples were obtained 0, 24, 48 and 96 h after RD treatment. The serial sections were stained with rabbit anti-GRP78/BiP antibody (ab21685, 1:1000, Abcam, Cambridge, MA, USA) and rabbit anti-Melan-A/MART-1 (NBP1-30151), consecutively, followed by staining with the appropriate secondary antibody (Nichirei). Immunoreactivity was detected with 3′-diaminobenzidine (Dako) for GRP78/BiP and aminoethyl carbazole (Nichirei) for Melan-A/MART1 followed by counter-staining with hematoxylin.

2.9. Statistical analysis

Significance of differences was calculated by Student's *t*-test (Microsoft Excel). A *p*-value of ≤ 0.05 was considered statistically significant.

3. Results

3.1. Establishment of transgenic mice with melanocytes in epidermis mimicking Japanese skin

In 1998, a membrane-bound/soluble stem cell factor (hk14-SCF) Tg mouse, which maintains epidermal melanocytes and pigment production, was developed by Kunisada et al., to study epidermal melanocytosis [10]. However, this Tg mouse had black skin (mean melanin index: 1000), and contained excess melanin to investigate RD-induced leukoderma in this study. Therefore, we developed new hk14-SCF Tg mice according to the previous method [9]; these mice had much lighter skin (mean melanin index: 500), similar to the color of Japanese skin. Furthermore, we crossbred the hk14-SCF Tg mouse with a hairless mouse (Hos:HRM) to develop a mouse with skin very similar to human skin, and lastly, we developed four mice models for Japanese skin. One of models was used in this study (Fig. 1A–C). Immunohistochemical analysis of skin specimens using an anti-Melan-A antibody showed Melan-A-positive melanocytes in the epidermis in quantities similar to that of human skin. In the process of establishing the newly developed mouse models, we also established a mouse model that retains Melan-A-positive melanocytes in the epidermis, similar to human albinism, but without tyrosinase activity in the melanocytes (Fig. 1 D–F).
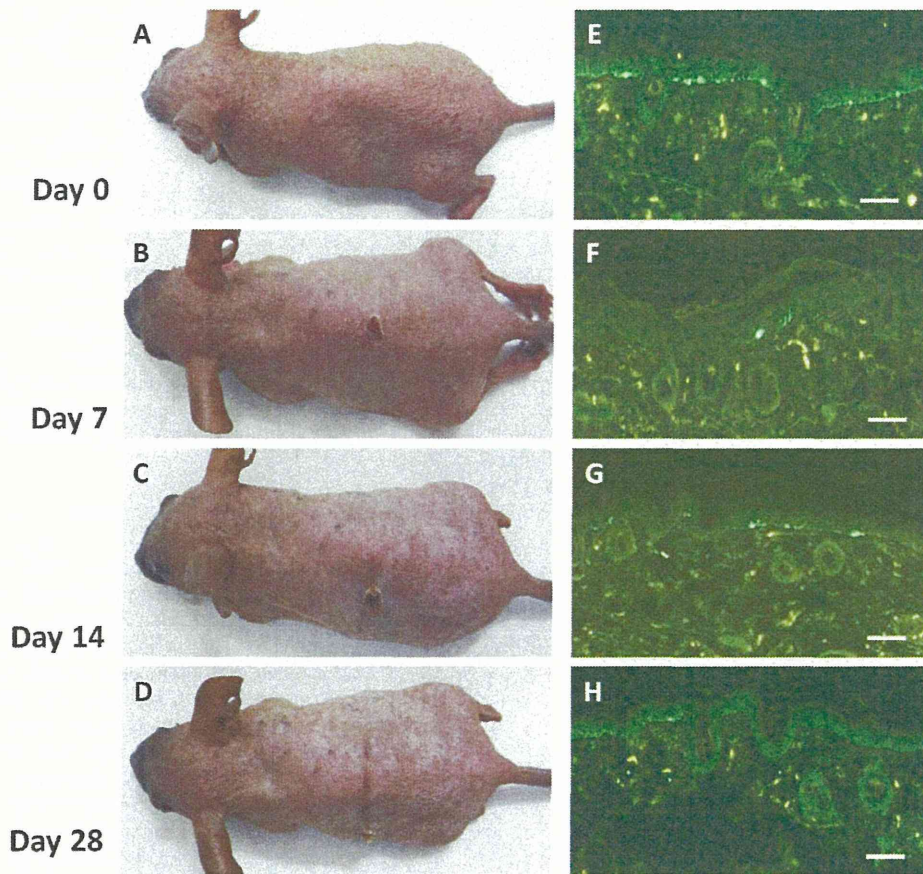
3.2. Induction of depigmentation by RD

To analyze the pathomechanisms of RD-induced leukoderma, we applied cosmetics containing 2% RD to the backs of the hairless hk14-SCF Tg mice for more than one month; however, no apparent leukoderma developed at the application sites. Then we prepared a 30% RD solution in 50% EtOH and a control of 50% EtOH. Two-hundred microliters of each solution was applied to a site on the backs of seven mice, respectively, three times per day according to a previous paper that reported induction of leukoderma on the back of guinea pigs [9]. On Day 7, the slight depigmentation was first observed at the sites treated with 30% RD (Fig. 2B), and on Day 14, marked depigmentation occurred (Fig. 2C). The mottled pattern on the back of the mice was very similar to that seen in human patients. With further treatment, the depigmentation gradually
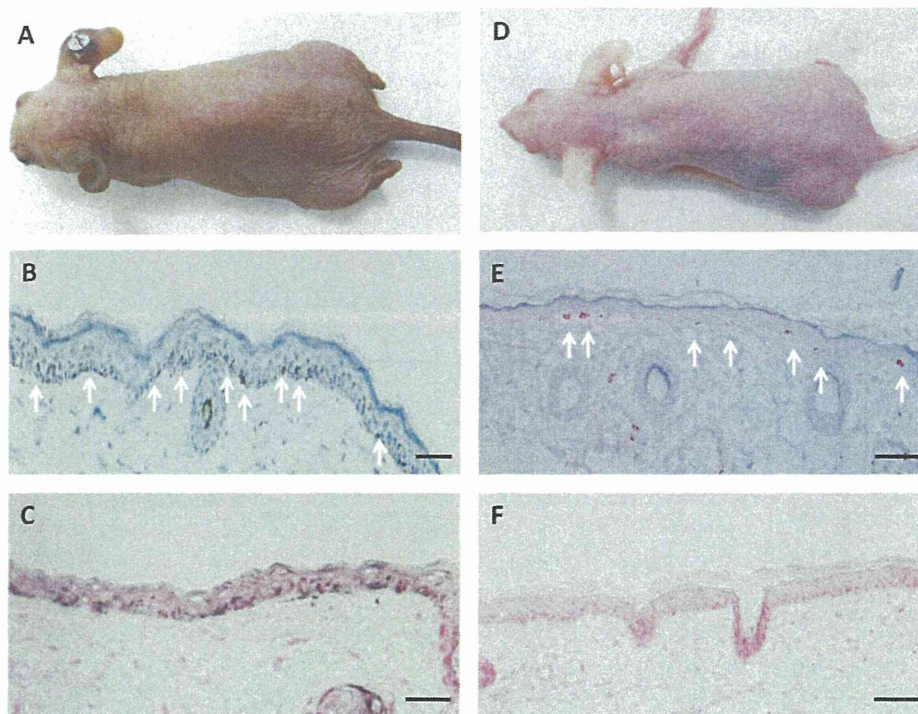
Fig. 1. Clinical and histological features of hairless hk14-SCF Tg mice and albino mice.(A) The hairless hk14-SCF Tg mice resembling Japanese skin color. (B) Distribution pattern of melanocytes (white arrows) as assessed by immunohistochemical staining. (C) Dopa-positive melanocytes. (D) The albino mice of the same strain. (E) Melan-A-positive melanocytes (white arrows) on albino mice. (F) Absence of dopa-positive melanocytes on albino mice. Bar indicates 50 μm.

Fig. 2. Depigmentation induced by RD in mice and immunohistochemical staining of RD-treated skin. (A) The skin color of Tg mice resembles Japanese skin color on Day 0. (B) A slight depigmentation of RD-treated sites was observed on Day 7. (C) Marked depigmentation was observed on Day 14. (D) Depigmentation gradually increased by Day 28. (E) Staining shows Tg mouse skin contains Melan-A-positive melanocytes. (F–H) Melanocyte concentration reduced from Days 7 to 28. Bar indicates 100 μm.

increased (Fig. 2A–D). On the final day, six of the seven mice displayed apparent depigmentation. As for skin color measurements, the melanin index decreased significantly at the area treated with 30% RD versus-melanin index of the control area, where 50% EtOH was applied between Day 7 and 28 (Fig. 3).

3.3. Decreased melanocyte count in the epidermis of pigmented mice treated with RD

To investigate the histopathological mechanism of leukoderma on the back of the hairless hk14-SCF Tg mice, we collected skin biopsy specimens from the leukoderma sites. The immunofluorescent
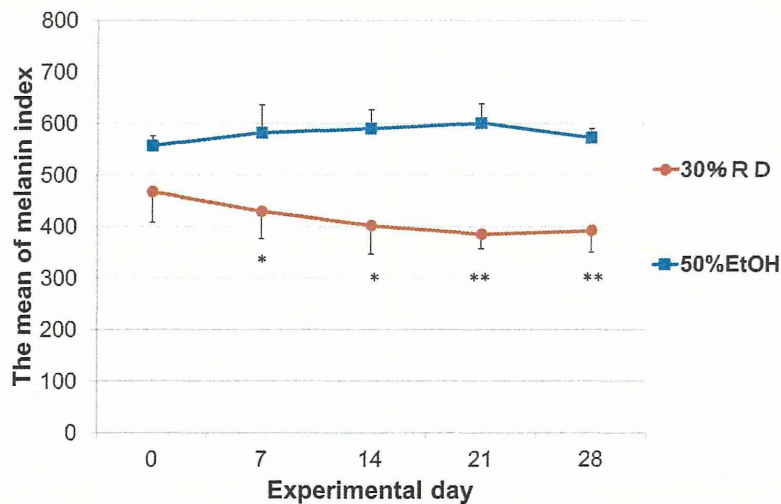
Fig. 3. The skin color of RD-treated area. The melanin index of the skin of RD-treated mice significantly decreased compared with that of EtOH-treated mice between Day 7 and 28 versus Day 0. *$p < 0.01$ and *$p < 0.001$ by Student's t-test.

analyses were performed with anti-Melan-A antibody. The results shown in Fig. 2E-H indicated that the number of Melan-A-positive melanocytes was decreased as early as Day 7 (Fig. 2F), and on Day 14, it was further reduced (Fig. 2G). Then, we counted the number of melanocytes in the epidermis. Fig. 4A shows the number of melanocytes per high-power field as identified by anti-Melan-A antibody staining. The number of Melan-A-positive melanocytes in the basal layer and hair follicles was significantly decreased from Day 7. But a few remained at Day 28 (Fig. 2H).

3.4. No difference in the number of melanocytes in albino mice treated with RD

Recent papers reported that tyrosinase activity was required for the melanocyte cytotoxicity caused by RD in the experiments using cultured melanocytes [4–9,11]. We investigated the application of this hypothesis to in vivo system using the albino mice established in this study. We applied two-hundred microliters of 30% RD solution on the back of five albino mice and counted the number of Melan-A-positive melanocytes in the epidermis. The result showed that melanocytes remained in the basal layer of the area treated with 30% RD, and no difference were found in the mean number of melanocytes between the 30% RD-treated group and the 50% EtOH control group (Fig. 4B), indicating that RD-induced cytotoxicity is dependent on cellular tyrosinase activity.

3.5. Contents of eumelanin, pheomelanin and metabolites of RD in the hairless hk14-SCF Tg mice

Previous papers reported that the metabolites of RD in cultured melanocytes were more cytotoxic than RD itself and would play a central role in the toxicity to melanocytes [6,8,9]. We investigated the metabolites of RD in the skin of the model mice. We analyzed the contents of eumelanin (EM), pheomelanin (PM), and RD-pheomelanin (RD-PM) in skin samples exposed to 30% RD for 0, 3,

7, and 15 days, using the methodology of Ito et al. [11]. The results showed that these mice produced melanin comprising mostly EM, with a low level of PM (Fig. 5A). After RD application, the level of EM in these mice gradually decreased. On the other hand, the level of PM slightly increased. RD-PM was not detected in the skin of the model mice. As for the metabolites of RD, the levels of non-protein thiol (GSH and cysteine) adducts of RD-quinone increased from Day 3 to Day 15 (Fig 5B). Free 5-S-cysteinyldopa (5SCD) and 5-S-glutathionyldopa (5SGD) are products of the addition of GSH and cysteine, respectively, to dopaquinone, the precursor of natural melanin. Free cysteinyl-RD-catechol (Cys-RDC) and glutathionyl-RD-catechol (GS-RDC) are products of the addition of GSH and cysteine, respectively, to RD-quinone [11]. Free RD-catechol (RDC) is also produced during tyrosinase oxidation of RD [4,6,7]. The protein-SH adduct of RD-quinone, measured as protein-bound Cys-RDC, was also detected in addition to the dopaquinone-protein adduct, measured as protein-bound 5SCD (Fig 5C).

3.6. Autophagy-lysosomes pathway and ER stress in melanocytes of mice treated with RD

We observed morphological changes in the melanocytes of the mice through electron microscopic evaluation of melanocytes of the model mice exposed to 30% RD. As shown in Fig. 6, double-membrane-walled structures containing electron-dense material, which might be typical for melanin-containing autophagosomes, on Day 4 (Fig. 6A), and a dilated ER, which would indicate ER stress [12], on Day 7 (Fig. 6B) were detected. These findings were not observed control mice (data not shown).

3.7. Melanocytes highly expressing GRP78/BiP tended to be increased by RD

We investigated the ER stress immunohistochemically. We took biopsy specimens from the leukoderma site at 0, 24, 48, and 96 h,
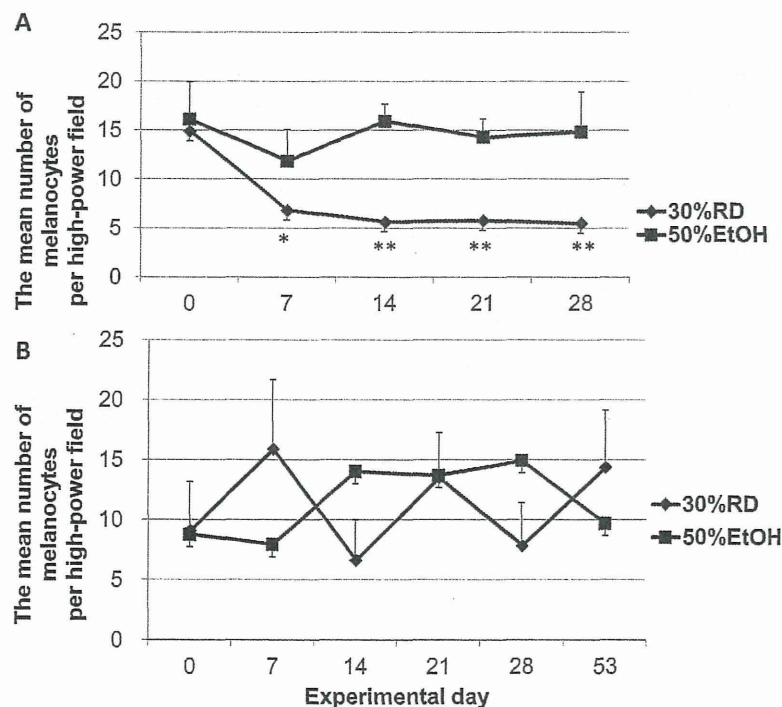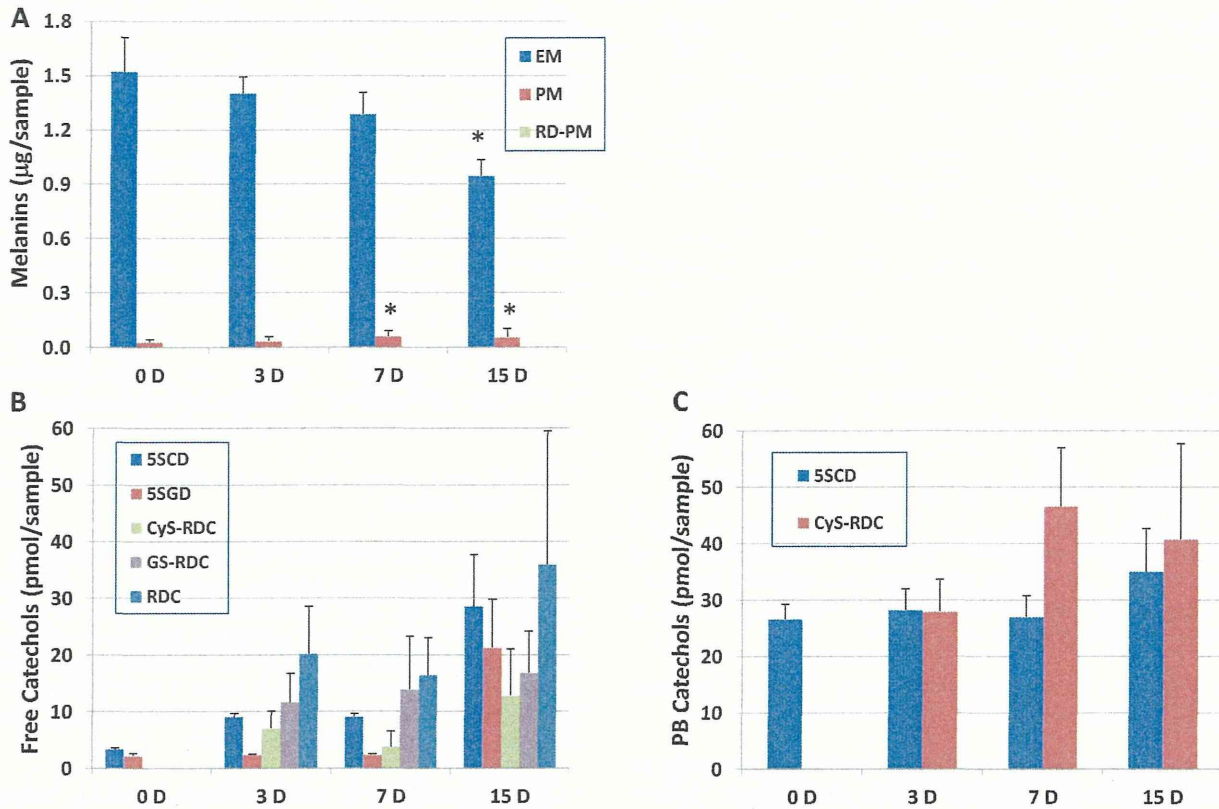

Fig. 4. The mean number of melanocytes per high-power field. (A) In hairless hk14-SCF Tg mice, the number of Melan-A-positive melanocytes significantly decreased from Day 7. *$p < 0.01$ and **$p < 0.001$ by Student's t-test. (B) There was no difference in the mean number of melanocytes in albino mice.

Fig. 5. Changes in the contents of eumelanin, pheomelanin, and adducts of non-protein thiol and protein-SH. (A) Changes in melanin contents. EM (eumelanin), PM (pheomelanin), and RD-PM were analyzed as described in Ref. [11]. RD-PM was not detected. (B) Changes in free catechols. 5SCD and 5SGD are thiol adducts of dopaquinone. Cys-RDC and GS-RDC are thiol adducts of RD-quinone. RDC is produced during the tyrosinase oxidation of RD. (C) Changes in protein-bound (PB) catechols. 5SCD and Cys-RDC are produced by HCl hydrolysis of the protein fraction of the skin. Protein-bound 5SCD and Cys-RDC are formed by addition of protein-SH to dopaquinone and RD-quinone, respectively. These metabolites were analyzed as described in ref. 11 and evaluated versus Day 0. *$p < 0.05$ by Student's t-test. Mean ± SEM for five mice.
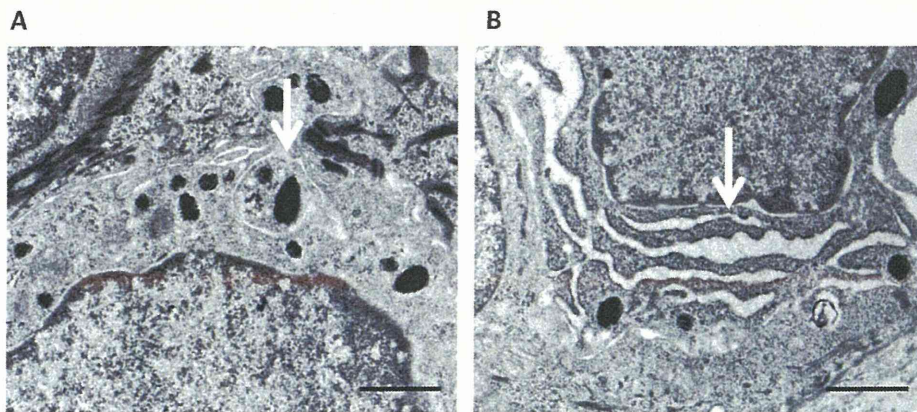
Fig. 6. Electron microscopy images of melanocytes exposed to 30% RD. (A) Arrow represents autophagosomes of double-membrane-walled structures containing electron-dense material (melanin or melanosome) on Day 4. (B) Arrow represents a dilated endoplasmic reticulum on Day 7. Bar indicates 1 μm.

and stained them with anti-GRP78/BiP and anti-Melan-A antibodies. GRP78/BiP is involved in the folding and assembly of proteins in the ER, and is a good marker for ER stress[13]. As shown in Fig. 7, melanocytes highly expressing GRP78/BiP were more frequently observed after RD treatment (24, 48, and 96 h in Fig. 7B, C, and D) than control (0 h in Fig. 7A). The proportions of GRP78/BiP highly expressing melanocytes per all melanocytes in basal layer were as follows: 23.5% (4/17) at 0 h, 41.4% (12/29) at 24 h, 40.7% (11/27) at 48 h, and 31.4% (16/51) at 96 h. These changes were not

observed in control mice receiving 50% EtOH (data not shown). The results showed that ER stress was activated in the melanocytes of the basal layer in the model mouse after RD treatment.

3.8. Recovery of depigmentation caused by RD

To examine whether re-pigmentation would occur at the leukoderma sites, the 30% RD application to the hairless hk14-SCF Tg mice was stopped after 28 days, and the mice were observed
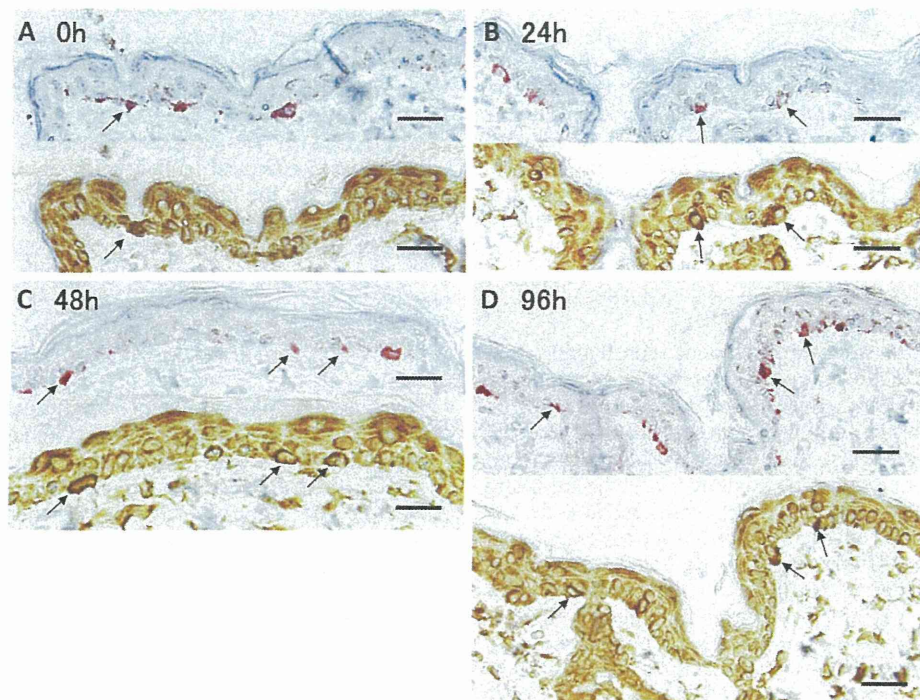
Fig. 7. Immunohistochemical images of Melan-A (upper panel) and GRP78/BiP (lower panel) evaluated by serial sections (4 μm) of 30% RD exposed mouse skin tissues (0, 24, 48 and 96 h after application in A, B, C, and D, respectively). Melanocytes with high expression of GRP78/BiP (arrows) were more frequently observed after RD treatment (24, 48, and 96 h in B–D). Bar indicates 30 μm.

until the pigmentation returned to the baseline level. After the treatment of RD, it took 42 days for re-pigmentation to return to the same level as found in untreated skin (Fig. 8A and B) and a few white spots remained. Skin specimens were obtained from both the re-pigmentation site and the white spot site of these mice, after 42 days. Immunofluorescent analysis showed the extension of a

significant number of dendrites on the melanocytes at the re-pigmentation site (Fig. 8C). This indicates that the melanocytes in the re-pigmentation site had been activated. On the other hand, no dendrites were observed in the melanocytes found in the white spots sites (Fig. 8D).
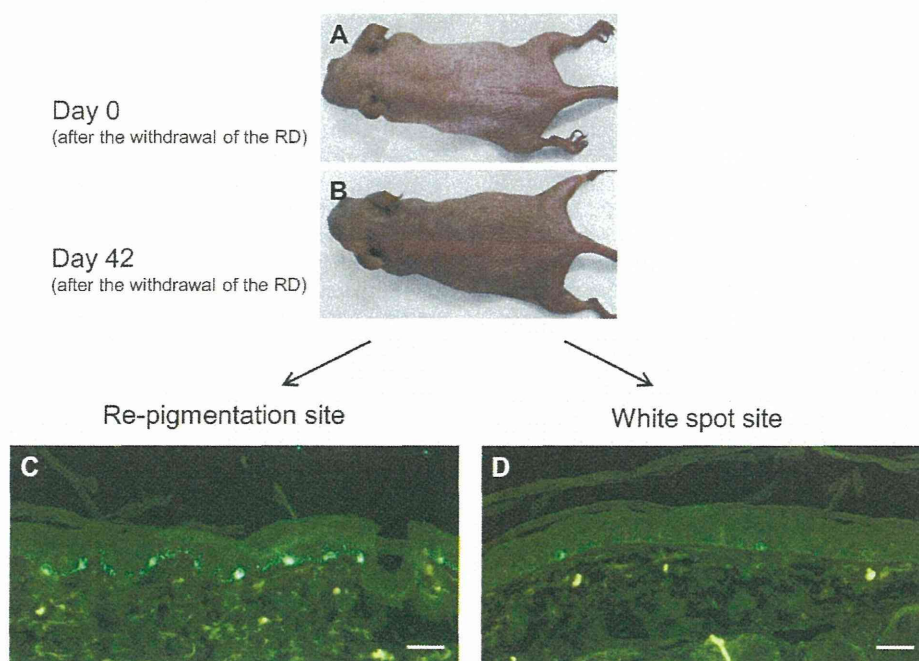
Fig. 8. Recovery from depigmentation caused by RD. Immunohistochemical staining of Melan-A. (A) Depigmented skin. (B) Re-pigmented skin on Day 42 after the withdrawal of the RD. (C) The melanocytes in the re-pigmentation site were actively dendritic. (D) The melanocytes at the white spot site were not dendritic. Bar indicates 30 μm.

4. Discussion

To investigate the pathomechanism of a disease, it is of great advantage to develop an animal model, because it is difficult to take biopsies and clinical samples frequently from patients. In this study, we successfully developed a mouse model of RD-induced leukoderma using hairless hk14-SCF Tg mice, with newly established and maintained melanocytes in the epidermis in a quantity similar to that found in human skin. After the treatment for seven days, this mouse model showed a mottled pattern on the back, resembling human RD-induced leukoderma (Fig. 2B–D). We used the mouse model to investigate the mechanism of RD-induced depigmentation in vivo.

We observed that the frequent continuous topical application (three times per day) of 30% RD on the backs of the mice induced depigmentation in six of seven mice. However, one mouse showed no depigmentation. It is interesting to note that not all RD-treated skin areas on the backs of the mice developed depigmentation; further, not all RD consumers have leukoderma, with the morbidity rate being 2% in humans [14]. Tyrosinase activity in the melanocytes might differ in each of the model mice, although the apparent skin color of each mouse was quite similar and not distinguishable from that of the others. Similarly, a threshold of tyrosinase activity was reported to determine melanocyte tolerance to RD in a tyrosinase-activity-dependent manner in vitro [7].

The reduction of the number of dopa-positive and Melan-A-positive epidermal melanocytes was observed in mouse model of RD-induced leukoderma. In contrast, the number of melanocytes was unaffected after application of 30% RD to the backs of the albino mice of the same strain, which had dopa-negative and Melan-A-positive epidermal melanocytes. These results provided evidence in vivo that the RD toxicity to the melanocytes was dependent on cellular tyrosinase activity, indicating that the tyrosinase would be the key molecule in RD-induced leukoderma. These observations are in agreement with those reported in previous studies using mushroom tyrosinase and cultured melanocytes [4–7,11].

In the biochemical analyses using the methodology recently reported [11], a reduction of EM was confirmed but RD-PM was not detected in the RD-treated skin samples of the model mice. Previously, RD-PM was detected in B16F1 melanoma cells exposed to 0.3–0.5 mM for 72 h [11]. The reason that RD-PM was below the detection limit in the mouse leukoderma skin was not clear; however, it may be related to the fact that PM was detected only at trace levels (Fig 5A). Higher levels of non-protein thiol addition products of RD-quinone and free Cys-RDC and GS-RDC were detected than the levels of dopaquinone products (Fig. 5B). This finding implies the following biochemical consequences: (1) glutathione and cysteine, two essential cellular antioxidants, are consumed through binding to RD-quinone, and (2) the products Cys-RDC and GS-RDC (and RDC itself) that are catechols may be cytotoxic through production of reactive oxygen species [4,6,7,11,15,16]. A Protein-SH addition product of RD-quinone, protein-bound Cys-RDC, was detected at levels comparable to the product of dopaquinone (Fig. 5C). The SH group (cysteinyl residue) is essential for the activities of many enzymes. Some examples include DNA polymerase α, thymidylate synthase, and isocitrate dehydrogenase. Therefore, the increased production of protein-bound catechol may lead to the suppression of cell growth and finally to cell death [11,16,17].

By electron microscopy, we observed autophagy-lysosomes and ER expansion in the cytosol of melanocytes. Also, using immunohistochemistry, we showed that the melanocytes in the leukoderma of the mouse model expressed GRP78/BiP, which is a good marker of ER stress [13], indicating the induction of ER stress by RD application. These data supported the previous report that RD activates the autophagy-lysosome pathway in melanocytes [8]. Based on these findings, it seems likely that a tyrosinase-dependent accumulation of RD metabolites linked to ER stress would contribute to the melanocyte cytotoxicity.

In the current study, Melan-A-specific CTLs are involved in the pathogenesis of RD-induced leukoderma as well as in other disseminated chemical leukodermatous lesions in patients with leukoderma on the unexposed areas [18]. Additionally, Nishioka et al. investigated T-cell populations in the lesional skin and peripheral blood of the RD-induced leukoderma patients [19]. They suggest that CTLs serve as inflammatory players in the lesional skin and imply that not only the toxic effect on melanocytes but also the immunological consequences further induce or exaggerate the depigmented lesions. And, some notable cases were reports of depigmentation caused by phenolic derivatives. Cutaneous depigmentation caused by phenolic derivatives was first described by Oliver et al. [20]. Additional reports have subsequently been published demonstrating occupational/contact vitiligo developing in people working with rubber [20] and industrial oils [21], paratertiary-butyphenol containing adhesives [22], and in the general manufacturing of these chemicals [23]. These studies have shown that the depigmentation extended from the site of chemical contact and developed into progressive, generalized vitiligo in almost 2% of individuals who were exposed to these chemicals [24]. This observation suggested that the response to these chemicals would be associated with a genetic variability [25]. The next steps would be to explore immunological mechanisms and genetic predisposition.

When the 30% RD solution was applied for 28 days to the backs of the hairless hk14-SCF Tg mice, the majority of melanocytes disappeared from the epidermis, but a few melanocytes remained in the basal layer and hair follicles, as determined by immunofluorescent analysis. It is quite possible that the remaining melanocytes would help the re-pigmentation in the basal layer and hair follicles, after the RD application is stopped. In fact, we observed re-pigmentation of the depigmented area in our model mice, which were withdrawn from RD after 42 days (Fig. 8). Furthermore, we very recently reported that re-pigmentation found in RD-induced leukoderma patients sometimes occurs from hair follicles in the same manner as that of vitiligo vulgaris [26].

In conclusion, we have developed hairless hk14-SCF Tg mice with skin color very similar to that of Japanese skin, by crossbreeding hairless mice and hk14-SCF Tg mice. Using both the hairless and the hk14-SCF Tg mice, we succeeded in developing RD-induced leukoderma on the backs of the mice. At the site of leukoderma, decreased number of melanocytes was observed, along with ER stress, decreased melanin levels, production of RD-metabolites, and dysregulated autophagy-lysosome pathway in the melanocytes. These results supported the results of many experiments in vitro which were recently reported [4,6–8,11], and presented direct evidence of the cytotoxicity of RD to melanocytes in epidermis in vivo. Going forward, because their biochemical and histological characteristics are closely similar to those of human skin, the newly generated mice from this study will be used as experimental animal models to investigate chemical vitiligo.

5. Conflicts of interest

The authors have no conflict of interest to declare.

6. Funding sources

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