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REVIEW ARTICLE

Rhododendrol-induced leukoderma update II: Pathophysiology, mechanisms, risk evaluation, and possible mechanism-based treatments in comparison with vitiligo

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Abstract

A small proportion of individuals utilizing cosmetics containing rhododendrol developed leukoderma with various pathological conditions, in some cases indistinguishable from vitiligo. In this review, we investigate and evaluate the major considerations for developing rhododendrol-induced leukoderma based on data from original or review articles published in the literature to provide a wide range of information regarding the pathophysiology, mechanisms, risk evaluation, and possible mechanism-based treatments. We compile and discuss the latest information, including data related to the cytotoxicity of rhododendrol, cytoprotective functions, and involvement of the immune system, and consider the possibility of novel treatments based on the differences between individual patients and on the mechanism underlying the onset of the condition. Understanding the pathophysiology of rhododendrol-induced leukoderma helps not only elucidate the mechanisms of non-segmental vitiligo onset and progression, but also suggests prevention and treatment.

KEYWORDS
leukoderma, melanocyte, oxidative stress, rhododendrol, vitiligo

1 | INTRODUCTION

The Japan Dermatological Association established the "Special Committee on the Safety of Cosmetics Containing Rhododenol (idiomatic name of rhododendrol)", chaired by Professor Kayoko Matsunaga, on July 17, 2013. In July 2016, the Rhododenol Research Team (RD-Team) was formed and commissioned by Kanebo Cosmetics to conduct research on the pathophysiology and treatment for rhododendrol (RD)-induced leukoderma (RDL), evaluate effective treatments from a medical standpoint, and provide information to a wide range of people. The present review article provides the latest information related to the pathophysiology and mechanism of RDL in comparison with generalized and progressive vitiligo, and further describes the risk assessment of RDL development and the possibility of new mechanism-based treatments, taking the diversity of development and symptoms into account. We also consider the factors underlying why only 2.4% of users have developed this condition.

2 | DIVERSITY OF CLINICAL FEATURES OF RDL

Approximately 98% of users of RD-containing cosmetics do not develop leukoderma. Conversely, 2.4% users developed leukoderma with various pathological conditions,[1] based on which the development and healing process could be roughly classified into three types:[2]

1. Cases in which leukoderma occurred only at the site of cosmetic application and improved spontaneously once the application of cosmetics was stopped, without treatment.
2. Cases in which leukoderma occurred only at the cosmetic application site and did not recover immediately after application of cosmetics was stopped, but showed improvement after treatment.
3. Cases in which leukoderma occurred in areas other than the cosmetic application site, sometimes expanding to non-application sites.

These findings suggest that multiple mechanisms may be involved in the development and healing processes of RDL, in addition to the factors that generate individual differences in the onset of the condition. In particular, the third class of cases appears to be indistinguishable from non-segmental vitiligo. For considering the pathogenic mechanism of RDL as well as vitiligo, it is helpful to compare RDL with vitiligo in terms of clinical features and *in vivo* and *in vitro* studies. Table 1 presents a brief summary of such comparison based on representative findings from patients in addition to *in vivo*/*in vitro* studies; the details are described in the following sections.

3 | MECHANISM OF RDL DEVELOPMENT

3.1 | Estimation of RDL onset mechanism based on histopathological analysis

Histopathological analysis of specimens of RDL-affected skin, showed pigmentary incontinence and residual melanocytes,[2] unlike the phenotypically similar disorder, vitiligo. Conversely, epidermal keratinocytes did not display notable abnormalities upon optical microscopic observation, whereas electron microscopic observation revealed residual melanocytes with degenerative melanosomes in the leukoderma lesions as a feature specific to RDL.[3] Therefore, RD was expected to exhibit melanocyte-specific cytotoxicity as the other organelles remained intact.[4]

Infiltration of $CD4^+$ and $CD8^+$ T cells was also observed in immunohistochemical analysis; however, unlike in vitiligo, $CD8^+$ T cells were not dominant, suggesting the involvement of an immune response different from that associated with vitiligo. Together, these findings suggest that RDL may be caused by melanocyte damage and local immunoreactivity distinct from vitiligo.

3.2 | Mechanism of melanocyte-specific cytotoxicity induced by RD

3.2.1 | Analysis of cytotoxicity and tyrosinase enzyme reaction using a cell culture system

Experiments using human melanocytes and mouse melanoma B16 cells, and analyses using tyrosinase enzymes have been performed, from which the following points were elucidated (Figure 1):

1. Specific knockdown of tyrosinase in cultured melanocytes reduces the cytotoxicity of RD.[5] Among melanocytes derived from different donors, cells with high dihydroxyphenylalanine (DOPA)-oxidizing activity show greater damage,[6] suggesting that melanocytes are damaged in a tyrosinase-dependent manner.
2. Kinetic analysis using RD as a substrate for mushroom tyrosinase,[5] and a series of studies on the absorption analysis of oxidized metabolites with tyrosinase,[7,8] demonstrated that RD (L-form, R-form, and racemic form) is a good substrate for tyrosinase, and that RD quinone generates oxidized products such as RD catechol. In these studies, RD quinone easily reacted to form 5-*S*-substituted adducts in the presence of thiol (SH) groups such as cysteine and glutathione, indicating the consumption of glutathione and attack of protein cysteine residues.[9] RD-oxidized metabolites were certainly detected in the *in vitro* enzyme reaction as well as in the human melanocyte and B16 cell culture systems.[6,10]
3. RD induces the endoplasmic reticulum (ER) stress response and apoptosis in melanocytes.[5,11–13]

TABLE 1 Comparison of rhododendrol (RD)-induced leukoderma (RDL) and vitiligo based on representative findings from patients and *in vivo*/*in vitro* studies

	Representative findings of patients					
	Depigmentation site	**Depigmentation spots**	**Repigmentation**	**Histological findings**	**Melanocytes (*in vitro*)**	**Animal models**
RDL	• Only at the site of cosmetic application	• Mixed incomplete and complete leukoderma • Partially mottled with relatively indistinct margins	• Improved without treatment after discontinuation of application	• Presence of residual melanocytes • Pigmentary incontinence in the dermis with melanophage infiltration • Predominance of $CD4^+$ rather than $CD8^+$ T cells	• No information regarding the characteristics of melanocytes from patients • Tyrosinase-dependent cytotoxicity induced by RD in normal human epidermal melanocytes • Cytoprotection by the NRF2 antioxidant system	• Tyrosinase-dependent melanocyte-specific depigmentation at the site of RD application • Improve without treatment after discontinuation of application • No involvement of autoreactive $CD8^+$ T cells
			• Improve as a result of the treatment, but difficult to distinguish from improvement resulting from the natural course of the condition			
	• In areas other than the application site, which sometimes expanded to non-application sites	• Complete leukoderma in the end • Indistinguishable from vitiligo	• Refractory	• Indistinguishable from vitiligo • Presence of Melan A-specific cytotoxic T cells		• No animal models for vitiligo induced by RD
Vitiligo	• Generalized and progressive	• Complete and patchy depigmentation	• Refractory	• Loss of melanocytes or detachment from basement membrane • Invasion of $CD8^+$ as well as $CD4^+$ T cells • Presence of melanocyte-specific autoreactive $CD8^+$ resident memory T cells (Trm)	• Sensitive to oxidative stress • Impaired signaling of NRF2-antioxidant responsive elements	• Depigmentation accompanied by accumulation of autoreactive $CD8^+$ T cells in the skin (an adoptive transfer mouse model)

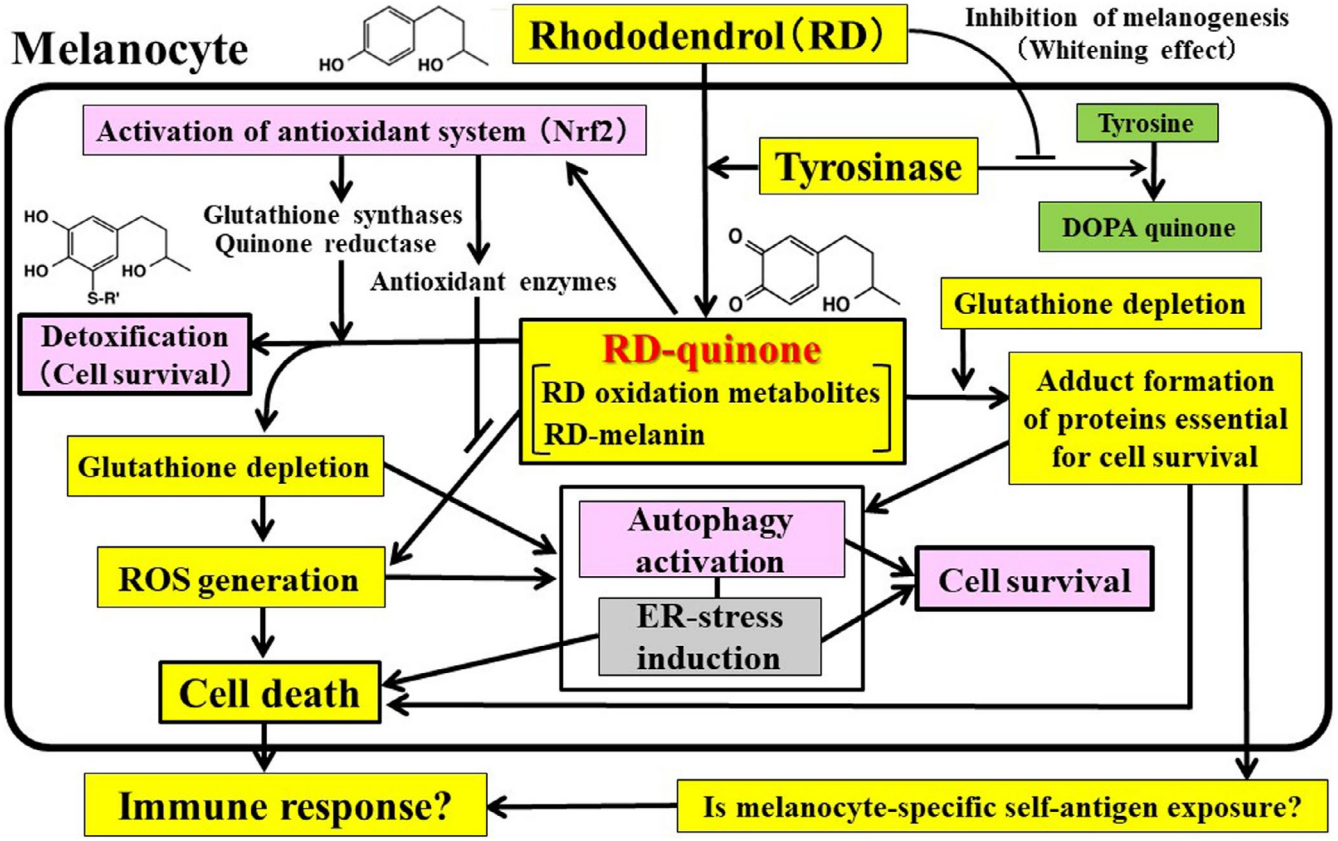

FIGURE 1 Mechanism underlying melanocyte-specific cytotoxicity induction and avoidance by rhododendrol (RD) (summary)

4. Reactive oxygen species (ROS) are generated during the process in which RD is oxidized by tyrosinase; or the process whereby oxidized metabolites undergo structural transformation via redox reaction.[12,14–17]
5. Ultraviolet (UV)-B light increases the content of RD quinone-derived products in melanocytes via tyrosinase activity and may act as an exacerbating factor of RD cytotoxicity by inducing ER stress.[18]

Furthermore, experiments using B16 cells and mouse models showed the formation of RD-oxidized products and reactive adducts with cysteine, glutathione, or cysteine residues of proteins, indicating that this reaction occurs *in vivo* as well.[10,19]

The non-enzymatic attack by the SH group on RD quinone to form a 5-*S*-substituted RD catechol rather than the RD cyclic quinone can be theoretically explained, based on the calculation of free energy using the first-principles calculation method.[20] In addition, RD eumelanin and pheomelanin are produced in B16 cells as tyrosinase metabolites. RD eumelanin, in particular, has been shown to oxidize cysteine, glutathione, and ascorbic acid, depleting these antioxidants and generating ROS.[21]

These results suggest that the generation of RD quinone, and the subsequent formation of oxidized metabolites by the tyrosinase activity, increases oxidative stress owing to glutathione consumption, inactivation of essential proteins for survival by cysteine residue attack, and generation of ROS, ultimately inducing cytotoxicity. In the *in vitro* tyrosinase reaction, generation of ROS can be confirmed when RD is used as a substrate;[17] however, ROS in cells may or may not be detected, depending on the cells or experiments used. In terms of cell death, both apoptosis and necrosis have been reported; therefore, the results may differ depending on the cells and culture conditions used, such as the RD treatment concentration and time.[5,12,14–16]

3.2.2 | Cytoprotective factors affecting RD toxicity

Another characteristic of the RD cytotoxicity ascertained in cell culture systems using melanocytes or melanoma cells is that the concentration of toxic RD varied significantly between experiments, occasionally by a difference of up to two orders of magnitude, resulting in an increase from the micromolar to the millimolar range.[5,6,11,12,14,16,22,23] Moreover, occasionally no cell damage was observed, depending on the cells or the culture density,[5,14] suggesting that this may be associated with a defense mechanism against RD cytotoxicity. The following research results have been reported:

1. Promotion of autophagy via rapamycin reduces RD cytotoxicity; conversely, inhibition of autophagy increases cytotoxicity.[11]
2. Increased activity of NAD(P)H: quinone oxidoreductase-1 (NQO-1), an antioxidant quinone reductase regulated by the transcription factor NRF2, reduces RD cytotoxicity.[22]

3. Increasing the intracellular glutathione pool or eliminating ROS using *N*-acetyl-L-cysteine and inducing the NRF2 system reduces RD cytotoxicity. Conversely, knocking down *NRF2* mRNA increases RD cytotoxicity.[16,23]
4. RD activates the melanocyte NRF2 or autophagy pathway.[11,23]

3.2.3 | Studies using RDL-induced animal models

It is crucial to experimentally reproduce RDL in animal models to elucidate its pathogenesis and pathophysiology, and to establish treatment methods based on research. In brown or black guinea pigs, which contain melanocytes in the epidermis similar to those in humans, continuous topical application of 30% RD three times a day for approximately 20 days induced significant depigmentation, in which a decrease in melanin content and the DOPA- and S100-positive epidermal melanocyte count remained. Skin color recovered and the number of melanocytes began to increase over 30 days after application ceased, suggesting that RD is cytotoxic to melanocytes and induces skin depigmentation. Moreover, as observed in several human cases, the depigmentation is reversed after discontinuation of RD application.[24]

Unlike guinea pigs, normal mice do not have epidermal melanocytes. Therefore, models using the hk14-stem cell factor (SCF) transgenic hairless mice, which contain epidermal melanocytes in the epidermis and have a skin tone similar to that of the Japanese skin type, were examined.[19] Application of 30% RD thrice daily induced depigmentation, with melanocyte loss on day 14. Conversely, the number of melanocytes did not decrease in albino mice, which lack tyrosinase activity. These results suggest that tyrosinase-dependent melanocyte-specific depigmentation can be reproduced. Furthermore, eumelanin levels decreased at the site of RD application, and RD quinone metabolites were formed. Additionally, electron microscopy of mouse skin revealed the existence of a bilayer structure, possibly resulting from the presence of melanin-containing autophagosomes, as well as that of a swollen endoplasmic reticulum, indicative of ER stress. These results suggest that the *in vitro* findings regarding RD-induced melanocyte-specific cytotoxicity were reproducible in mouse models.[19]

Given that autoreactive $CD8^+$ T cells were observed in the RDL, and its pathology may be related to an autoimmune mechanism, the effect of a Janus kinase (JAK) inhibitor (tofacitinib) on RDL was investigated using the SCF transgenic mice, as above;[25] however, no significant difference was observed between the two groups following the p.o. administration of tofacitinib 25 mg/kg/day or the vehicle, suggesting that JAK inhibitors are not involved in the promotion of pigment regeneration or melanocyte migration.

3.3 | Involvement of the immune system in RD-induced melanocyte loss

Tyrosinase peptide-specific cytotoxic T cells have been suggested to be involved in a melanocyte-specific cytotoxic mechanism.[26] When T-cell lines were established from peripheral blood monocytes derived from eight patients with RDL (seven of whom were $HLA\text{-}DR4^+$), a tyrosinase peptide-specific T-cell response, restricted to major histocompatibility complex (MHC) classes I and II, was observed. It is assumed that the antigenicity of tyrosinase peptide is altered to a non-natural form upon binding to RD (a concealed antigen), and MHC-restricted cytotoxic T cells may specifically attack melanocytes in the presence of RD.[26] However, there remain certain unresolved issues, particularly with respect to the limited number of cases, and it is unknown whether the concealed tyrosinase antigen is actually presented on melanocytes. In addition, the relationship between leukoderma symptoms and tyrosinase reactivity remains poorly understood.

Although $CD8^+$ T cells did not appear to be dominant in the histopathological analysis of RDL,[3] clinical cases exist where leukoderma occurred in areas other than the application site.[2] Therefore, the immune system, which has been thought to be important in vitiligo, was suspected to be involved in these cases. In fact, immunosuppressants (such as tacrolimus) have been effective in some cases. Thus, a possible mechanism underlying in this condition may involve RD damage to melanocytes, which induces an immunological response and promotes lymphocyte infiltration in the affected area.

An analysis of RDL patients revealed an increase in Melan-A-specific cytotoxic T cells (CTL) in $HLA\text{-}A^*02{:}01^+$ cases, suggesting that damaged melanocyte-specific protein fragments are recognized by antigen-presenting cells, inducing self-antigen-recognizing CTL.[27] Notably, RDL at non-RD-application sites exhibits clinical symptoms similar to those of vitiligo; moreover, Melan-A-specific CTL may be involved in the spread of vitiligo induced by other chemicals, as well as in RDL (Figure 2). However, it cannot be ruled out that the 28 patients included in this report were selected as patients who had a refractory spread of depigmentation and were originally vitiligo.

CCR4, a receptor for chemokines CCL17 and CCL22, that is required for T-cell homing to the skin, is primarily expressed on $CD4^+$ T cells and is a marker for T-helper (Th)2 cells, but is also expressed on $CD8^+$ T cells. In particular, $CD8^+$ T cells in the blood from patients with non-segmented vitiligo express CCR4 more frequently

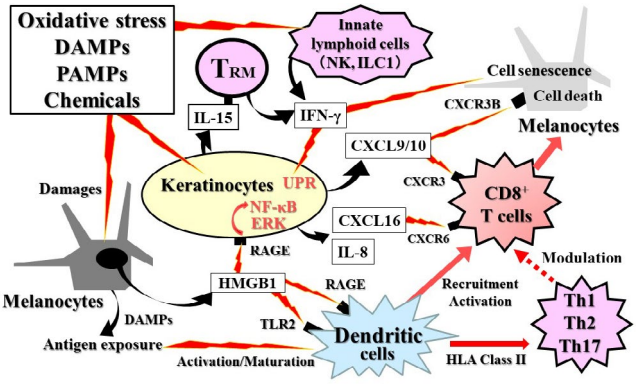

FIGURE 2 Possible involvement of innate and acquired immune systems in the development and maintenance of vitiligo

than in those from healthy individuals.[28] Nishioka *et al*.[29] reported that the T-cell ratios of $CCR4^+$ to $CD8^+$ are significantly higher in peripheral blood and skin tissues from patients with RDL and vitiligo than in those from healthy individuals, and the ratios in peripheral blood decreased over time following discontinuation of RD usage. The authors also suggests that CCL22 and CCL17 are involved in RDL, and macrophages are involved in the homing of $CCR4^+$ $CD8^+$ T cells to the skin.[29]

Conversely, when comparing the serum levels of autoantibodies such as anti-thyroid peroxidase in RDL patients who did not improve after discontinuing use of cosmetics and those who improved, no statistical correlation was shown between the presence of autoantibodies and lack of improvement in skin symptoms. Moreover, although a limited number of patients were analyzed, a significant number of autoantibodies were detected in the serum of patients of vitiligo but not RDL. This suggests that the factors involved in the onset of vitiligo and RDL may differ.[30]

In addition, it has recently been reported that the anti-programmed death 1 antibody, a checkpoint blockade drug, causes vitiligo, possibly owing to an enhanced autoimmune response to melanocytes. Thus, additional pathways may also need to be considered for the immunological aspects of RDL.[31]

3.4 | Involvement of other cell types in RD-induced melanocyte loss

The involvement of keratinocytes and fibroblasts has been reported in the melanocyte elimination mechanism in vitiligo.[32] In turn, cells such as mast cells, dendritic cells, and keratinocytes may be involved in the loss of melanocytes and in excessive pigment regeneration in RDL, as observed in vitiligo as well.[33]

3.4.1 | Keratinocytes

In the pathogenesis of vitiligo, the response of keratinocytes as recipients of cytokines and danger-associated molecular patterns (DAMP) released when the skin is subjected to various endogenous and exogenous stress factors, ultimately leads to vitiligo. For example, interferon (IFN)-γ and the nuclear protein HMGB1 stimulate keratinocytes to produce chemokines (CXCL9/10, CXCL16, and interleukin [IL]-8, among others), which allow $CD8^+$ T cells to migrate to the skin and induce antigen-specific melanocyte damage (Figure 2).[34,35]

Genome-scale transcriptional analysis of human keratinocytes treated with a non-cytotoxic hydroquinone (HQ) revealed that HQ significantly upregulated genes associated with the IL-17 signaling pathway, whereas it significantly downregulated those associated with melanogenesis.[36] Both HQ and IL-17 induce keratinocyte IL-36γ production, and directly inhibit melanogenesis in human epidermal melanocytes; hence, the ability of other vitiligo risk substances to induce keratinocyte IL-36γ production would be notable.

A recently proposed mechanism for the disappearance of melanocytes in vitiligo lesions involves the disappearance of E-cadherin from melanocytes in the lesion, which decreases adhesion to keratinocytes, leading to the melanocytes being excreted transepidermally.[32] Abnormalities in adhesion molecules, other than E-cadherin in keratinocytes at the transition and lesion area of vitiligo, may be associated with adhesion between keratinocytes, melanocytes, and the basement membrane.

The glycoprotein non-metastatic melanoma protein B (GPNMB), a membrane protein known to be expressed in melanocytes and involved in processes such as melanosome formation, stress resistance, and cell adhesion, is expressed in cultured human keratinocytes and in the basal layer of healthy epidermis, whereas it is absent in basal layer lesions in vitiligo.[37] Notably, it was confirmed that IFN-γ and IL-17A, which are thought to be involved in the development of vitiligo, suppress keratinocyte GPNMB expression.[37] It has also been found that keratinocyte GPNMB was absent in basal layer lesions in RDL, and that the extracellular domain of GPNMB is released as soluble GPNMB, which protects melanocytes against cytotoxicity (unpubl. data, Professor Katayama, Department of Pigmentation Research and Therapeutics, Osaka City University, Osaka, Japan), suggesting that keratinocyte GPNMB may be involved in vitiligo and RDL pathology.

3.4.2 | Resident memory T cells (T_{RM})

A useful model was recently proposed in which the specific chemokine CXCL16 is produced via an unfolded protein response in epidermal keratinocytes during oxidative stress, and $CD103^+$ and $CD49a^+$ T_{RM} cells are activated to impair melanocytes.[38] This has garnered attention as a new model of vitiligo pathogenesis, which links oxidative stress and the induction of autoimmune responses. An etiology has been proposed whereby ROR (retinoic acid receptor-related orphan receptor)-γt-induced Th17-type T_{RM} is involved in psoriasis, whereas in vitiligo IL-15 is activated and Th1-type T_{RM} cells are involved, as they produce cytotoxic factors such as IFN-γ, granzymes, and perforin. This discovery is expected to aid the development of novel therapeutic agents. Similarly, RD may also induce oxidative stress in keratinocytes (Figure 2).[38]

3.4.3 | Innate lymphocytes

A recent influential theory in the pathogenesis of vitiligo proposed that IFN-γ stimulates keratinocytes to produce CXCL10, which allows $CD8^+$ T cells to migrate to the skin and cause antigen-specific melanocyte damage.[34] Consistent with this, the efficacy of a JAK inhibitor that blocks IFN-γ signaling in vitiligo has been reported.[39] Although several aspects of the origin of epidermal IFN-γ remain unclear, epidermal-resident type 1 innate lymphoid cells were recently reported to produce IFN-γ in response to endogenous and exogenous stress stimuli (Figure 2).[40] This process may thus be involved

in the development of chemical-induced vitiligo by phenolic compounds, including RD.

3.4.4 | Mast cells

Skin mast cells proliferate in an SCF (or KIT-ligand)-dependent manner; however, c-kit, the receptor for such mast cells, is also present on melanocytes. c-kit is reportedly upregulated in keratinocytes in vitiligo lesions,[41] whereas another report claimed that it is downregulated.[42] In turn, Kotobuki *et al*.[43] reported that Th17 cells infiltrate the vitiligo-affected area, and that Th17 cell-derived cytokines, such as IL-17A, play an important role in inducing vitiligo. Recent reports have indicated that mast cells may produce IL-17 in psoriasis,[44] and may also be associated with vitiligo lesions and RDL.

Yasuda *et al*.[45] reported that the number of degranulated mast cells significantly increased in RDL lesions, and that mast cell-derived factors, such as IFN-γ and IL-17, were possibly involved in the onset, progress, and maintenance of vitiligo.[37] In recent years, cells such as natural killer (NK) cells and type 1 lymphoid cells are assumed to be produced in the skin (Figure 2).[40] It has been previously recognized that human mast cells have NK cell-like markers and functions.[46] It has also been reported that connective tissue-type mast cells amplify the Th1 immune response via STAT4,[47] and it was suggested that skin mast cells may be involved as IFN-γ-producing cells in either innate or acquired vitiligo.

3.4.5 | Langerhans cells

It has been reported that when $CD1a^+$ Langerhans cells become enlarged, their dendrites elongate, and the cells migrate to the basal layer of the epidermis in vitiligo lesions.[48] The same phenomenon has been reported in RDL. Notably, the Langerhans cells are distributed at the same density as the original melanocytes in locations where the melanocytes are eliminated; therefore, the Langerhans cells possibly migrate to melanocyte-anchoring sites in the basal layer (proposed as the melanocyte niche) and inhibit melanocyte anchoring.

3.5 | Mechanism of hyperpigmentation during pigment regeneration

Reportedly, histamine derived from mast cells, induces UV-induced pigmentation via histamine H_2 receptors in melanocytes, and histamine H_2 receptor antagonists inhibit increased pigmentation.[49] Additionally, it has been reported that the mast cell-derived PAR2 activator (tryptase) is involved in melanosome transfer from melanocytes to keratinocytes, and is also involved in melanin cap formation.[50] This suggests that mast cells may compensate for the reduced melanocyte activity in vitiligo, and may be involved in the increased pigmentation at the periphery of vitiligo lesions. However, the possibility remains that enzymes such as tryptase act on melanocytes in a disruptive manner. Mast cell-derived factors, such as histamine, may also be involved in the increased pigmentation at the margin of leukoderma lesions, as observed in RDL.

3.6 | Factors that caused individual differences in RDL development

The incidence of RDL is 2.4%; in most cases, the pigment regenerated immediately after discontinuation of RD application, although certain cases were noted in which the lesions spread to or developed in non-application sites after discontinuation.[2] Therefore, it is assumed that there are specific background factors that contribute to melanocyte dysfunction resulting from RD use. The following background factors were considered based on analyses of the aforementioned mechanisms.

3.6.1 | Environmental and genetic factors that elicit high tyrosinase activity

Mature tyrosinase in melanosomes is essential for the production of RD quinone, which triggers melanocyte cytotoxicity (Figure 1),[5] and RD must be efficiently incorporated into melanosomes to act as a substrate. Tyrosinase-inducing factors such as UV light and cytokines are thought to contribute to this process.[13,18] In addition, a recent genome-wide association study for 147 cases and 112 controls identified CDH13/T-cadherin as the strongest RDL susceptibility gene associated with tyrosinase expression as well as apoptotic pathway regulation.[51]

3.6.2 | Detoxification of RD quinone and ROS-capturing ability

The resulting RD quinone is detoxified through a non-enzymatic reaction with intracellular glutathione or cysteine in melanosomes or by enzymatic reduction. Therefore, the intracellular glutathione or cysteine pool, the ability to activate NRF2, which activates the glutathione detoxification pathway, and NQO-1 levels exert a major effect on RD cytotoxicity (Figure 1).[16,22,23]

The results of immunohistochemical studies on skin tissues from patients with RDL whose condition improved or was intractable revealed that in the improved cases, the expression of the glutamate-cysteine ligase catalytic (GCLC) subunit, a glutathione synthase, increased in melanocytes surrounding the leukoderma site.[45] However, there was no such increase in intractable cases.[45] Therefore, low GCLC levels and an insufficient supply of glutathione may constitute one of the factors that differs between improved and intractable cases of leukoderma. The ability to capture ROS generated by RD quinone may also affect RD cytotoxicity.

3.6.3 | Antigen-presenting ability of melanocyte-specific proteins

When melanin-specific protein fragments produced by intracellular RD damage are presented on MHC class I or II, they are attacked by cytotoxic T cells, resulting in melanocyte damage. Accordingly, it has been proposed that HLA-A*02:01 and HLA-DR4 are high risk factors for RDL development (Figures 1 and 2).[26,27]

The aforementioned genetic or environmental factors may be responsible for individual differences that determine whether leukoderma is induced by RD use as well as for the various individual differences in the pigment regeneration process after discontinuation of use. Further studies are required to investigate the differences in risk factors for chemical-induced leukoderma and vitiligo.

4 | EVALUATION OF RISK SUBSTANCES THAT MAY CAUSE CHEMICAL-INDUCED VITILIGO

Oxidative stress is considered as an environmental risk factor in the development of chemical-induced leukoderma as well as vitiligo. When considering the risk of developing chemical-induced vitiligo, it is important to evaluate, in advance, the substances that may cause leukoderma, and to use the results to form a comprehensive risk assessment.

4.1 | Risk evaluation using *in vitro* studies or cultured cells

Given that cytotoxicity is triggered by the tyrosinase-mediated conversion of RD to RD quinone, there are two primary methods for risk evaluation: method 1 evaluates whether RD quinone is formed (i.e., whether it becomes a substrate) using mushroom or human-derived tyrosinase;[8] and method 2 evaluates whether the cells show cytotoxicity using melanocytes or melanoma cells.[12]

An issue with using tyrosinase, as in method 1, is that mushroom tyrosinase has a very broad substrate specificity and can react with substances that are not considered to pose the risk of chemical-induced leukoderma or vitiligo. Conversely, although human tyrosinase may have a high substrate specificity owing to the narrow inhibitor spectrum of phenolic compounds,[52] this method poses a challenge because of the difficulty of preparation. Tyrosine is a natural substrate, and is abundant in living organisms; therefore, it is necessary to establish a complex evaluation criterion, including transfer into melanocytes and melanosomes, affinity with tyrosinase, and the reactivity (stability) of the quinone formed in the presence of physiological concentrations of tyrosine.

In turn, the most significant challenge to cell-based evaluation (method 2) is the difficulty in standardizing the evaluation results (e.g., reproducibility, fluctuation of cytotoxicity owing to cell culture conditions, and difference in results depending on the cells). In fact, reported RD melanocyte cytotoxic IC_{50} values range from concentrations of a few μmol/L to more than 10 mmol/L.[5,6,11,12,14,16,22,23] This is because the presence of defense functions in cells, typified by the NRF2 and ER stress response systems, and fluctuations in tyrosinase activity, which contribute to quinone production, constitute a significant and integral part of the evaluation results. Thus, it is necessary to establish a standard cell line wherein these factors can be controlled, and preferably, establish an evaluation method that will give uniform results, irrespective of the site of evaluation.

4.2 | Risk evaluation using animals

As mentioned previously, it was possible to induce leukoderma through the daily application of 30% RD in evaluation systems using brown and black guinea pigs with epidermal melanocytes, and using hk14-SCF transgenic hairless mice with skin color similar to the Japanese skin type. Moreover, Iida *et al*.[53] reported an evaluation system using mouse tail skin. The authors noted that the epidermal melanin density and the number of melanocytes in the mouse tail were greater by more than 50 and 4.5 times, respectively, than those in the dorsal skin. Therefore, they evaluated the risk of chemical-induced leukoderma caused by RD and raspberry ketone, which has a similar structure. They confirmed mild yet detectable depigmentation after 4 weeks of application of the drug at a concentration of 2% and confirmed a reduction in melanin levels as well as in the number of $TRP\text{-}2^{+}$ melanocytes by 14 weeks. Although this is proposed as a suitable risk assessment method that does not require UV irradiation or genetic modification, it is considered to be a model for evaluating chemical-induced leukoderma where pigment regeneration is observed after discontinuing application. Further verification is required to determine the reversibility of depigmentation, and in similar substances that have not been reported for vitiligo, to enable the use of this method as a risk assessment system.

As mentioned above, only partial evaluation of substances that pose a risk of inducing chemical-induced leukoderma or vitiligo is possible in both *in vitro* and *in vivo* systems. Moreover, there are currently no signs of progress in the establishment of an evaluation system involving the immune system.

5 | FUTURE DIRECTIONS WITH CONCLUDING REMARKS

Several agents have potential as novel treatments, based on the mechanism underlying RD development described in the text and the latest findings regarding vitiligo. These include signal transduction inhibitors,[54,55] biologics,[56,57] or promising application of epidermal cells[58–61] and stem cells, such as iPS cells and Muse cells to regenerative medicine for vitiligo treatment.[62,63] Now, the investigation of blood biomarkers and international standardization of treatment evaluation methods are being in progress conducted by Vitiligo Global Issues Consensus Conference Workshop (VGICC).[64] After enough discussion and evaluation by global members, new therapies will be introduced and applied to refractory RD patients.

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CONFLICT OF INTEREST

This research was founded by Kanebo Cosmetics. There are no other conflicts of interest to declare.

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