発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
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Abe Y, Okamura K, Kawaguchi M, Hozumi Y, Aoki H, Kunisada T, Ito S, Wakamatsu K, Matsunaga K, Suzuki T	Rhododenol-induced leukoderma in a mouse model mimicking Japanese skin.	*J Dermatol Sci.*	81	35-43	2016

Ⅳ．研究成果の刊行物・別刷

化学物質による白斑
—職業性白斑の機序とロドデノール白斑—

最上（西巻）知子

Leukoderma caused by chemicals: mechanisms underlying 4-alkyl/aryl-substituted phenols- and rhododendrol-induced melanocyte loss

Tomoko Nishimaki-Mogami

Chemical leukoderma is a skin depigmentation disorder known to occur in manufactural workplace through contact with chemicals, such as monobenzyl ether of hydroquinone (MBEH) and 4-tert-butylphenol (4-TBP). In the skin depigmented legions induced by these chemicals, the number of melanocyte was severely decreased. Anti-melanoma agent 4-cysteaminylphenol (4-SCAP) and its derivatives are also known to cause leukoderma. Evidence has accumulated supporting that typical class of chemicals causing leukoderma is "4-alkyl/aryl-substituted phenols/catechols", which are structurally similar to melanin precursor tyrosine. Tyrosinase-mediated oxidation of these chemicals yields toxic ortho-quinones which bind to cellular proteins and produce reactive oxygen species. Accordingly, this tyrosinase-dependent metabolic activation is thought to cause melanocyte-specific damage and subsequent immune reactions toward melanocytes. Recently, rhododendrol, an inhibitor of tyrosinase developed for so-called lightening/whitening cosmetics, was shown to cause leukoderma in the users. In this review, I document the causes of known chemical leukoderma and rhododendrol-induced leukoderma, focusing on their common mechanisms underlying melanocyte loss.

Keywords: Chemical leukoderma, monobenzyl ether of hydroquinone, rododendrol, melanocyte, tyrosinase

はじめに

メラニン生成抑制物質であるロドデノール（4-(4-ヒドロキシフェニル)-2-ブタノール, rhododendrol, Rhododenol）（図1）を配合した薬用化粧品の使用者の皮膚適用部位に「白斑」が多発することが判明し，2013年7月より製品の自主回収が行われた．製造販売業者の調査により，2014年7月31日の時点で19073人が発症し[1]，使用者数80万人に基づく発症率は約2%と報告されている[2]．

皮膚色素であるメラニンは，チロシナーゼを初発酵素とする経路においてチロシンより合成される（図2）．メラニンの生成を抑える化粧品成分，いわゆる「美白」成分の開発が盛んに行われている．ロドデノールもその一つで，チロシナーゼを競合的に阻害してメラニン生成を抑制する医薬部外品原料として2008年に承認され，2%配合製品が6年間販売された．ロドデノール白斑の患者においては，メラニン色素低下から完全消失まで様々な程度の脱色素斑が認められ[3]，病理組織所見では色素産生細胞であるメラノサイトの減少・消失が報告された[4,5]．同様の白斑/脱色素斑病変は，後天性疾患である「尋常性白斑」や，工場労働者などが化学物質に接触・曝露することにより発症する「職業性白斑」において認められている．この総説では，化学物質による白斑とその機序について過去の報告を紹介するとともに，ロドデノールにより引き起こされた白斑との関係について2015年5月まで論文発表された報告をもとに考察した．

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Tomoko Nishimaki-Mogami; Division of Biochemistry, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan; Tel:+81-3-3700-1141 ext.271; Fax:+81-3-3707-6950; Email: mogami@nihs.go.jp
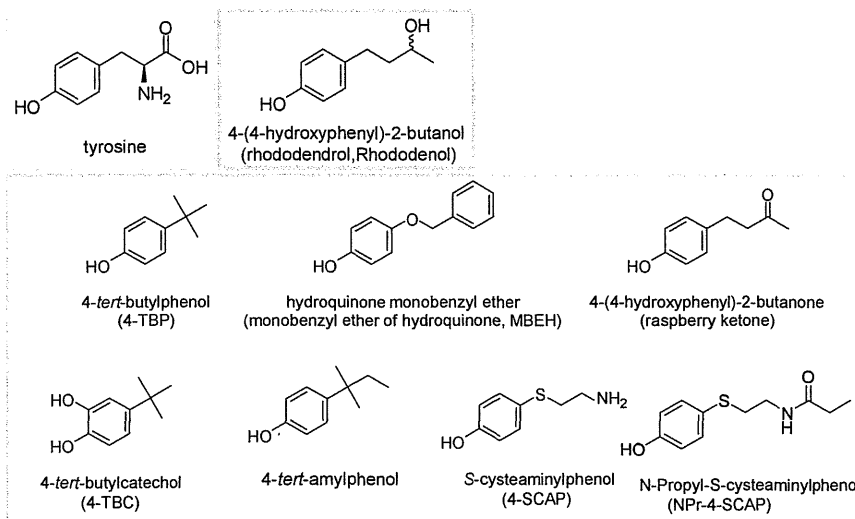
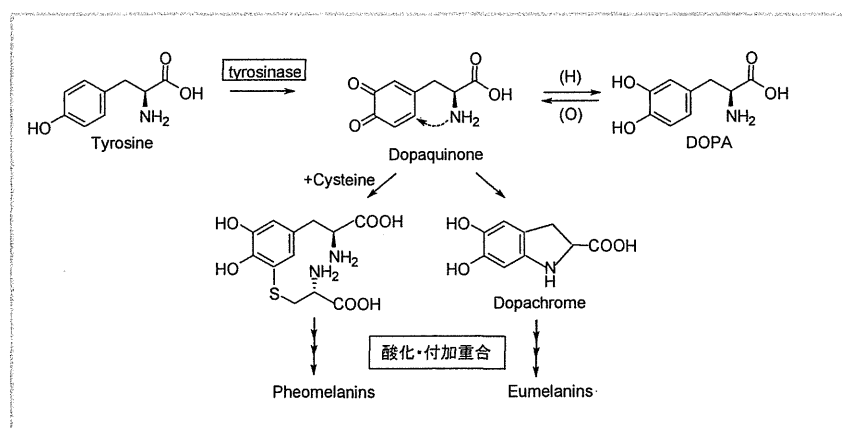図1 チロシン，ロドデノール，および職業性白斑や皮膚色素脱失を引き起こす化合物
(引用文献6-8, 10-12, 48, 49, 54-56)

図2 メラニン生合成経路

チロシナーゼによりチロシンから生じるドーパキノンは，分子内閉環を受けたのち，酸化・付加重合を繰り返して暗褐色～黒色のユウメラニンが合成される．あるいはシステインとの付加反応を受け，酸化・付加付加重合により黄色～赤褐色のフェオメラニンに転換される．

1．化学物質による白斑

1-1 4-置換フェノール/カテコール類による白斑/脱色素斑形成

化学物質により皮膚に白斑/脱色素斑が生じた例としては，1939年ゴム手袋に含まれるヒドロキノンモノベンジルエーテル（モノベンゾン，MBEH）（図1）に曝露された工場労働者の48人中25人に白斑が発症した事例[6]，英国で4-tert-ブチルフェノール（4-TBP）を扱う工場労働者198人のうち54人が発症し，1977年にLancetに報告された事例などが知られている[7]．このような職業性白斑はフェノール誘導体に大量曝露された人に高頻度に発生しており，動物実験を含む文献調査の結果，皮膚のメラノサイトを選択的に減少，あるいは消失させて白斑・脱色素斑形成を誘導する化学物質の代表的なクラスが，チロシンに似た構造を持つフェノール化合物である「パラ位に脂肪族/芳香族側鎖を持つフェノール・カテコール類」であると結論されている[6,8,9]（図1）．加えて，メラノーマ（悪性黒色腫）に対する抗ガン剤として開発研究が進められた4-S-システアミニルフェノール（4-SCAP）やその誘導体NPr-4-SCAP（図1）はこの定義に合致する化学構造を有しており，黒色マウスや黒色モルモットに皮下注射や塗布を行うと，皮膚や毛包メラノサイトの消失と毛色の脱色が生じることが報告されている[10-12]．

1-2 メラノサイト選択的傷害と二相性の機序

MBEHや4-TBP，NPr-4-SCAPなど4-(アルキル/アリル)置換フェノール類による皮膚白斑/脱色素斑病変部においては，メラノサイトが選択的に減少・消失している．メラノサイトの消失は，メラノサイトへの直接傷害に加え，免疫系を介する二相性の作用機序によることを示唆

する知見が得られている[12,13].

1-3 メラノサイトの直接傷害－チロシナーゼによる代謝活性化と酸化ストレス

4-TBP，MBEHやNPr-4-SCAPによるメラノサイトへの直接傷害には，チロシナーゼにはじまるメラニン合成経路が大きく関わることが，in vitroならびにin vivo研究において示唆されている．これらの化合物は，皮膚角化細胞（ケラチノサイト）に比較し，チロシナーゼを発現するメラノサイトに，より高い毒性を示す[14,15]．また4S-CAPやその誘導体NAc-4-SCAPやNPr-4-SCAPは，メラニン生合成の活発なメラノーマ細胞に，より強い毒性を示す[12,16,17]．In vivoにおいても，抗メラノーマ薬候補の4-SCAPは有色実験動物のメラノサイトを減少させる一方，チロシナーゼ活性を欠くアルビノマウスのメラノサイトやケラチノサイトには影響しないことが報告されている[10].

1-3-1 チロシナーゼによる代謝活性化

4-TBP，MBEHやNPr-4-SCAPなどの4-（アルキル/アリル）置換フェノール類はチロシンに似た構造を持ち，チロシナーゼを阻害しメラニン合成を低下させるとともに[18,19]，チロシナーゼにより代謝されオルトキノン体を生じる．これらのオルトキノン体は反応性が高く，システインやグルタチオン，チロシナーゼのSH残基に付加反応する[19-22]．図3に4-TBPならびにMBEHの例を示した．

オルトキノン体や付加体はさらなる代謝に伴い，セミキノンフリーラジカルやパーオキサイド，活性酸素種ROSを産生して細胞を傷害する機序が提唱されている[8]．実際に4-TBP，MBEH，NPr-4-SCAP処理メラノサイトではROS産生が促進されることが確認されており[12,19,23]，酸化ストレス増加がメラノサイト選択的な毒性発現をもたらすと考えられている．

1-3-2 メラニン合成系：酸化ストレス亢進と防御

チロシナーゼにはじまるメラニン合成経路は，酸化ストレス産生との大きな関わりが知られている．チロシナーゼが内因性基質であるチロシンを酸化して産生するドーパキノンは，システインのSH基とMichael付加反応してシステイニルドーパに転換され，さらに付加重合が繰り返されることにより黄色～赤褐色のフェオメラニンが合成される[24]（図2）．ドーパキノンがシステインSH基と反応して生じるフェオメラニン生合成中間体はROS産生を促進し，酸化ストレス増大により毒性を発揮することが知られている[25]．一方，ドーパキノンが分子内のアミノ基付加反応によりインドールに環化し，重合する経路に入ることにより暗褐色～黒色のユウメラニンが生じる．ユウメラニン合成経路の中間体は，酸化ストレスに対してむしろ保護的に働くとされ，ユウメラニン合成経路を不活化したマウスでは，酸化的傷害を介する発がんが亢進することが報告されている[26].
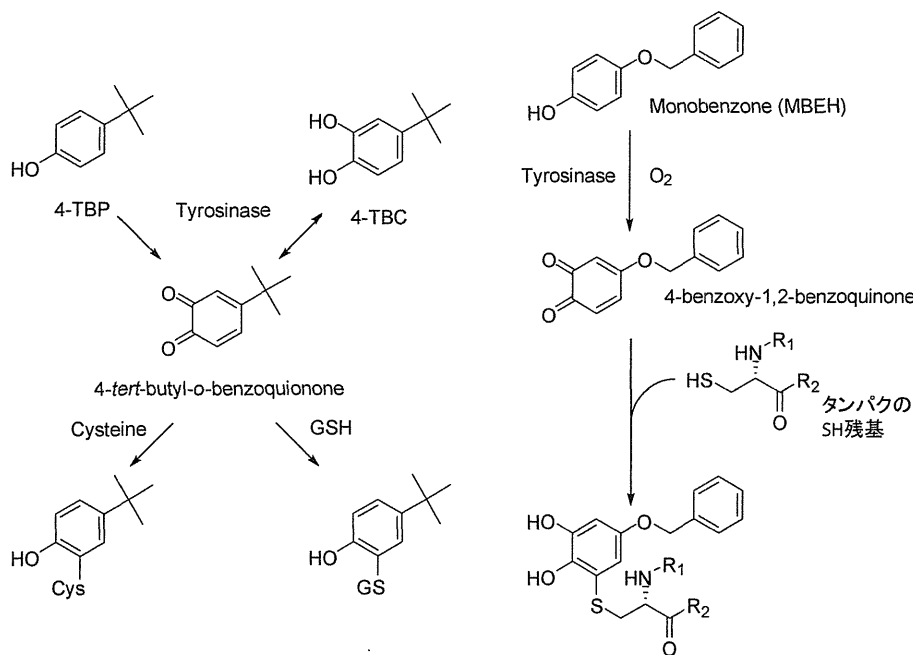

図3 4-TBP，MBEHのオルトキノン体への代謝とSH基との反応
4-TBPやMBEHはチロシナーゼにより代謝される．生成するオルトキノン体は反応性に富み，付加反応によりシステインやグルタチオン，タンパクのSH残基を修飾する．（引用文献19-21）

1-3-3 酸化ストレスとメラノサイトの感受性

　ROS産生による細胞傷害は，遺伝的に酸化ストレスに弱いメラノサイト，例えば細胞内のラジカルスカベンジ系に欠陥を持つようなメラノサイトでは増強されることが予想される．実際に，尋常性白斑の患者では表皮メラノサイトにおける過酸化水素レベルが高く[27]，過酸化水素を分解するカタラーゼを培地に添加すると4-TBPによる細胞毒性が低減する[23]．また4-SCAPのメラノーマ殺傷作用もカタラーゼ添加で抑制される[28]．したがって，4置換フェノール類による酸化ストレスが引き金となって白斑を発症するには，酸化ストレスに対するメラノサイトの耐性/感受性が大きく関わると考えられる[8,23]．

　4-TBPやMBEHはチロシナーゼにより代謝され，ROS産生を促進するが，意外なことに，4-TBPやMBEHによる細胞毒性はチロシナーゼ欠損や過剰発現により影響されず，チロシナーゼ活性との相関は認められない[14,19]．しかしながらこれらの細胞毒性はメラニン合成系と強く関係することが明らかにされている．4-TBPの細胞毒性は，メラニン合成の下流でインドールキノン産生に関わる酵素，チロシナーゼ関連タンパク質1（TYRP1）の過剰発現により増強される[8,23]．MBEHの感受性は逆に細胞内のメラニン量増加に伴い低下しており，ユウメラニン合成が酸化ストレスに対して保護的に働くことが感受性に影響する可能性が推定されている[15]．

1-4 メラノサイト傷害への免疫系の関与

　4-TBPやMBEH，NPr-4-SCAPによる皮膚メラノサイトの消失には，メラノサイトへの直接傷害に加えて，免疫応答やメラノサイトに対する自己免疫誘導も大きな役割を持つことが明らかにされている[12,29,30]．

1-4-1 チロシナーゼ代謝によるハプテン形成

　これらの4置換フェノール類はチロシナーゼにより活性代謝物であるオルトキノン体に代謝され，タンパク付加体を形成する（図3）．4-TBP[20]，MBEH[21]，4-SCAP[31]やその誘導体NPr-4-SCAP[22]はグルタチオンやタンパクのSH基にMichael付加する反応性を獲得し，MBEH[19]はチロシナーゼのSH基を修飾することが確認されている（図3）．このようなタンパク修飾体がハプテン抗原として提示され，ハプテン特異的あるいはメラノサイト特異的な免疫応答が誘導される機序が提唱されている[13]．

1-4-2 免疫系による応答の増強

　MBEHや4-TBPのメラノサイトに対する毒性は強力ではなく，IC$_{50}$は数百はμMのレベルである．しかしながら樹状細胞とのコミュニケーションにより，4-TBPがメラノサイトにアポトーシスを誘導する感受性が増大する[32]．

　また，MEBHおよび4-TBPはメラノサイト生存率に大きく影響しない濃度で小胞体ストレス応答（unfolded protein response：UPR）を誘導し，免疫応答増強につながるサイトカインIL-6・IL-8産生を誘導することが報告されている[30]．

1-4-3 自己免疫の誘導

　白斑には様々な病因の疾患が含まれるが，頻度の高い尋常性白斑は，現在では自己免疫性の疾患と認識されている．環境要因や生理的要因が引き金となって局所的なメラノサイトの死をもたらし，メラノサイト抗原が提示され，自己免疫応答を引き起こす．その結果，メラノサイトの破壊は全身に，離れた場所にも起こる[29,33]．患者の皮膚脱色部位にはT細胞が浸潤している[34]．

　4-TBPやMBEHによる職業性白斑の事例においても，白斑は曝露された人全てには発症せず，化合物に接触した部位以外にも広がる例が認められることから，化学物質は引き金となる環境要因の一つであり，メラノサイト傷害により自己免疫が誘導され，メラノサイトが破壊される機序が推定されている[8,29,33]．

　MBEHはいわゆる美白効果を目的として使用した健常人にも白斑症を引き起こすが，尋常性白斑の患者において，脱色素斑を目立たなくするために正常な皮膚を脱色素する薬としてFDAに認可されている[35]．MBEHを塗布した直後の脱色は局所に起こるが，数ヶ月後には予期せぬ場所に起こる場合が知られている．脱色素部位の皮膚にはCD8陽性の細胞傷害性T細胞が浸潤しており，メラノサイトが特異的に攻撃される自己免疫の誘導が示唆されている[13,33,35]．MBEHを皮膚に塗布するとメラノサイト特異的な免疫応答を誘導することは，マウスにおいても確認されている[36]．また，メラノーマ細胞を移植したマウスをNPr-4-SCAPで治療すると，メラノーマを再移植した場合においても増殖が抑制された．増殖抑制は細胞傷害性T細胞を介しており，同時に尋常性白斑様の脱色素斑が生じたことから，メラノーマ/メラノサイト特異的免疫が誘導されたことが示唆される[12]．

　このようにMBEHなどの化学物質が自己免疫応答を誘導するメカニズムとして，MBEHによるメラノサイトの死が抗原提示を促進する可能性が提唱されている[37]．また，MBEH代謝物がチロシナーゼを修飾するに伴い，メラノサイト抗原を含むエクソソームがメラノサイトから放出されることが報告されている[19]．職業性白斑の事例においては，遺伝的な要因が感受性の違い/個人差をもたらすと考えられ[8]，尋常性白斑に関連が見いだされた遺伝子の多型やMHC（HLA）クラスIの解析が，化学物質による白斑発症の原因究明の手がかりになる可能性も大きいと考えられる．

1-4-4　皮膚感作性試験とチロシナーゼ/メラノサイト

　遅延型アレルギーの可能性を検討する試験において，メラノサイトやチロシナーゼとの関わりが示唆されている．4-TBPはモルモットを使った感作性試験GPMT（Guinea Pig Maximization Test）において陰性であったが，4-TBPのチロシナーゼによる代謝で生じる4-tert-butylcatechol（4-TBC）は強力な感作性と4-TBPとの交差反応性を示した[38]．MBEHについては，ヒト（健常人）でパッチテストを行うと陽性は1.2%であったが，皮膚色素過剰症患者に2〜6ヶ月塗布すると13%の人に感作性を示し，皮膚炎を発症した[39]．白斑症の患者に塗布すると有色部に皮膚炎を発症し，パッチテストでメラノサイトが存在する部位にのみ炎症を起こすことが報告されている[40]．

2．ロドデノールによる白斑

2-1　臨床報告

　2013年7月に日本皮膚科学会に特別委員会が設置され，症例の疫学・臨床学的調査が行われた．一次全国疫学調査（1338人）においては，96%に製品使用部位に概ね一致した白斑/脱色素斑が認められた[3]．二次全国疫学調査の結果（2014年1月），やや軽快以上が72%で，全体の34%については脱色素斑面積が1/2以下になった[41]．多くの例では使用中止により徐々に色素再生が見られたが，一部では進行性や遠隔部位での白斑が報告された[4,42]．特別委員会三次全国疫学調査（2014年12月）において，対象患者の16%が使用中止後も脱色素斑が回復傾向を示さないか，あるいは悪化しており，非塗布部への拡大増悪例も認められることが報告された[43]．

　ロドデノールによる白斑/脱色素斑の成因として，メラノサイトの直接傷害に加え，免疫応答を介するメラノサイト消失機序を示唆する知見が報告されている．皮膚脱色素斑病変部では，メラノサイトが減少・消失し，基底層下にメラノソーム（色素顆粒）を貪食した繊維芽細胞が認められた[45]．病変部にはCD4陽性やCD8陽性T細胞など多様な免疫細胞が浸潤しており[5]，さらなる解析により，ロドデノール白斑患者においては，尋常性白斑の場合と同様に，血中ならびに皮膚病変部においてケモカインCCL22と，ケモカイン受容体CCR4を発現するCD8陽性T細胞が有意に高いことが判明した[44]．また，HLA型のHLA-A*02:01保有者では，メラノサイト抗原Melan-A-特異的なCD8陽性細胞傷害性T細胞が高頻度に検出されることが判明した[45]．したがって，メラノサイト消失には，ロドデノールによるメラノサイトの直接傷害に加えて，メラノサイトを特異的に攻撃する細胞傷害性T細胞が関わる場合が示唆される[46]．

2-2　実験動物での確認

　ロドデノールの皮膚色素脱失作用は，実験動物で確認されている．褐色モルモットの皮膚に高濃度のロドデノールを塗布すると，皮膚基底層のメラノサイトやメラニン顆粒が減少して色素脱失を形成すること，作用は可逆的であることが，花王の研究グループにより確認された[47]．一方，C57BL/6など黒色のマウスにおいても，メラノサイトは主として毛包に局在し，皮膚にはきわめて少ない．そこで皮膚と毛包に恒常的にメラノサイトを有するトランスジェニックマウスが作成され，ロドデノール経皮投与による白色化，メラノサイトの減少と回復が
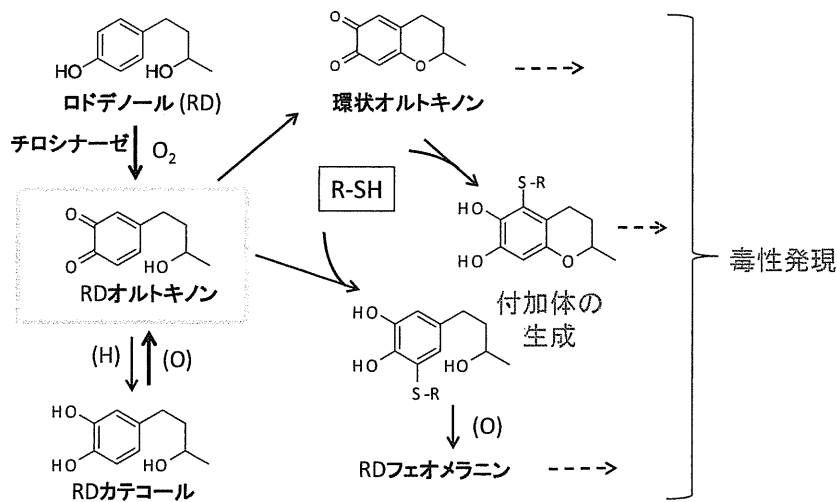

図4　ロドデノールのチロシナーゼによる代謝活性化

ロドデノールはチロシナーゼにより，オルトキノン体に代謝される．オルトキノン体は反応性が高く，環化，H_2O付加するほか，システイン・グルタチオンやタンパクのSH基に付加反応する．システインが付加すると，フェオメラニン様の色素が生成する．これらの代謝物は活性酸素種の生成を介して，毒性を発揮することが推定されている．（引用文献50−52）

学会発表されている.

　可逆的な皮膚色素脱失は，ロドデノールと構造が類似する4-TBP[48]やMBEH[49]でも報告されているが，過去の報告は黒色モルモットが使われており，また塗布方法・溶剤など条件が異なることから，ロドデノールと強度の比較は難しい.

2-3　ロドデノールのチロシナーゼによる代謝活性化 と細胞毒性発現

　ロドデノールはチロシナーゼ阻害によるメラニン生成抑制剤として開発されたが，チロシナーゼにより代謝され，毒性発現に関わる活性代謝物に転換されることが明らかにされている.　藤田保健衛生大学の伊藤らは，ロドデノールは4-TBP，MBEH，4-SCAPなど白斑誘導性4-置換フェノール類と同様に，チロシナーゼにより代謝されてオルトキノン体に活性化され，システインやグルタチオンなどのSH化合物に付加反応すること[50]（図4），オルトキノンへの代謝活性化はヒトチロシナーゼを用いても効率的に進行することを明らかにした[51].　オルトキノン体はまた，タンパクを修飾することが確認され[52]，チロシナーゼによる代謝活性化によりハプテンを形成する可能性が示された.

　生成するオルトキノン体は活性酸素種ROS産生を介して毒性発現に関わる可能性が推定されている[50].　オルトキノン体の非酵素的還元により生じるカテコール体4-(3,4-ジヒドロキシフェニル)-2-ブタノール（図4，RDカテコール）は，容易に自動酸化されてオルトキノン体に戻る.　カテコール体をヒトメラノサイトに添加すると，強力にROS産生を促進し，ロドデノールに比較しはるかに強力な細胞毒性を発揮した[2].

　実際に，ロドデノールのメラノサイトに対する毒性は細胞のチロシナーゼ活性に大きく依存することが，カネボウ・花王の研究グループにより示されている[2,53].　ヒトメラノサイトのロドデノール感受性は細胞の由来により大きく異なり，13株のメラノサイトは高感受性（IC$_{50}$値：0.14〜0.54mM）と低感受性（IC$_{50}$値：5.54〜6.8mM）の二群に分かれ，前者はチロシナーゼ活性が高く，後者は低い傾向が認められた[53].　さらに高感受性細胞のチロシナーゼをsiRNAノックダウン，あるいはフェニルチオウレアで阻害すると，IC$_{50}$値は低感受性細胞の域まで増加し，毒性発現へのチロシナーゼの関与が明確に示された[2].　またロドデノールは，チロシナーゼ活性に依存して小胞体ストレス応答を誘導することも報告された[2].

　このように，ロドデノールはチロシナーゼにより活性代謝物オルトキノン体に転換されること[50,51]，チロシナーゼと毒性発現との大きな関わりが明らかにされたことから[2,53]，チロシナーゼによる代謝がメラノサイト選択的傷

害をもたらすことが強く示唆される.　このようなメラノサイトの傷害が引き金となり，メラノサイトに対する免疫応答が誘導される可能性が推定される.

　ヒトメラノサイトは個人差が大きく[53]，ロドデノールに高感受性を示す細胞の入手が困難である.　いわゆる美白剤の安全性評価には，チロシナーゼによる代謝活性化測定法を確立すること，ならびにロドデノール感受性に関わる因子・白斑発症に強く関連する因子を明らかにし，代替細胞系を確立することが必要と考えられる.

3．まとめ

　職業性白斑の原因物質MBEHや4-TBP，抗メラノーマ薬候補4-SCAPなど，メラニン前駆体チロシンに構造が似た化学物質「パラ位に脂肪族/芳香族側鎖を持つフェノール類」は，ヒトや実験動物において皮膚の接触部位に白斑様症状を誘導することが知られる.　これらフェノール類はチロシナーゼにより酸化され，細胞タンパクに結合するとともにROSを産生する毒性物質，オルトキノンを生じる.　このようなチロシナーゼによる代謝活性化がメラノサイト特異的な傷害を引き起こし，引き続きメラノサイトに対する免疫応答の誘導により，メラノサイトが選択的に減少・消失して白斑を発症すると考えられている.

　配合化粧品による白斑が問題化したロドデノールは，美白成分として開発されたチロシナーゼ阻害剤であるが，上記フェノール類と同様に，チロシナーゼにより活性代謝物オルトキノン体に転換され，システインやタンパクのSH基を修飾することが判明した.　ロドデノールはチロシナーゼ活性に依存してメラノサイトに毒性を発現することが実証され，毒性代謝物によるメラノサイト傷害が示唆されている.

　臨床において，ロドデノール白斑患者の多くは徐々に色素再生が見られたが，一部の患者では増悪や非塗布部への拡大が認められた.　病変部や血中に免疫細胞が増加し，メラノサイトを特異的に攻撃する細胞傷害性T細胞が検出される例から，メラノサイトの消失には，直接傷害に加えて，メラノサイトに対する免疫応答が関わることが示唆される.　したがって，ロドデノールは，化学構造，皮膚の白斑/脱色素斑の病理，in vitroでの代謝・細胞毒性発現，直接ならびに免疫を介するメラノサイト消失・白斑形成機序の全てにおいて，上記フェノール類と極めて類似すると判断される.

　医薬部外品・化粧品成分による健康被害防止のためには，これらの化学物質による白斑発症に重要な因子と評価に有用な指標を明らかにし，評価試験系を確立することが急務と考えられる.

本総説は，平成25-26年度厚生労働科学研究「ロドデ
ノール配合薬用化粧品による白斑症状の原因究明・再発
防止に係る研究」分担研究「原因究明に関する調査研究
I. ロドデノールおよび類似化学物質による白斑症状に
関する文献調査」（分担研究者：最上知子）の成果に基
づき執筆した．

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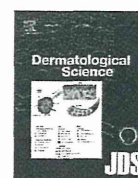56) Solano F, Briganti S, Picardo M, Ghanem G: *Pigment Cell Res.* 2006;19:550-71.

Letter to the Editor

4-(4-Hydroroxyphenyl)-2-butanol (rhododendrol) activates the autophagy-lysosome pathway in melanocytes: Insights into the mechanisms of rhododendrol-induced leukoderma

Rhododendrol [4-(4-hydroxyphenyl)-2-butanol, Rhododenol[®]] was first developed 5 years ago as a skin-whitening cosmetic that contained a 2% (w/w) formulation. It is also a natural ingredient present in many plants, such as the Nikko maple tree [1]. It competes with tyrosine for hydroxylation by tyrosinase and effectively inhibits melanin biosynthesis [2,3]. However, these cosmetics were withdrawn from the market in 2013 after rhododendrol reportedly caused a depigmentation disorder. It was reported that partial depigmentation appeared on the neck, the hands, and the face at sites of repeated application of these cosmetics, and the symptoms in 79% of affected patients disappear or begin to improve within 6 months after ceasing product use [The Japanese Dermatological Association Special Committee on the Safety of Cosmetics Containing Rhododendrol, 2014 (in Japanese)]. A previous report suggested that the melanocyte toxicity of rhododendrol is caused by its tyrosinase-catalyzed oxidation and production of cytotoxic reactive oxygen species (ROS) [4]. Another previous study reported that no ROS were detected in rhododendrol-treated melanocytes, but a tyrosinase-dependent accumulation of endoplasmic reticulum (ER) stress and/or activation of the apoptotic pathway appeared to contribute to cytotoxicity [5]. However, contradictorily, it was also recently reported that one melanocyte line with higher tyrosinase activity is resistant to rhododendrol cytotoxicity [6]. Furthermore, depigmentation symptoms have only been confirmed in ≈16,000 (2%) of 800,000 estimated users of cosmetic products containing rhododendrol. Interestingly, not all consumers and not all treated skin areas developed white blotching. It is still unclear why only some people suffer from these skin problems and why a spectrum of symptoms occurs. It appears likely that rhododendrol causes irritation and depigmentation disorders only under certain conditions, suggesting that its mechanisms might be more complicated than previously reported.

In this report, normal human epidermal melanocytes from moderately pigmented neonatal foreskin (HEMn-MP) cells were incubated with rhododendrol (300–3000 μM). Rhododendrol treatment at 300–900 μM did not affect melanocyte viability. However, the number of viable melanocytes strikingly decreased in response to rhododendrol treatment at 1500 and 3000 μM (Fig. 1A). These results demonstrated concentration-dependent rhododendrol-induced cytotoxicity in melanocytes. After rhododendrol treatment, cultured melanocytes were washed with cold PBS buffer, adherent cells were lysed for western blotting analysis (Fig. S1A) or were fixed for immunofluorescence staining (Fig. S1B). Treatment with higher concentrations (1500–3000 μM) of rhododendrol induced caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavages in melanocytes (Fig. S1A). The cleaved caspase-3 positive melanocytes were also observed after treatment with high concentration of rhododendrol (Fig. S1B). All of these results together suggest that rhododendrol induces apoptotic cell death in HEMn-MPs.

Cultured HEMn-MPs were treated with non-cytotoxic rhododendrol concentrations (300–900 μM) for 120 h. Rhododendrol-treated cells were markedly less pigmented than untreated control melanocytes (Fig. 1B(a–c)). By western blotting, identical treatment with rhododendrol markedly down-regulated expression of the melanogenic factors tyrosinase, Pmel17 and Melan-A, while slightly increasing MITF expression (Fig. 1B(d)). By western blotting, expression of the autophagosome marker LC3II and lysosome marker LAMP1 were both markedly up-regulated in melanocytes by rhododendrol treatment at non-toxic concentrations (Fig. 1C). Furthermore, by immunofluorescence staining, increased LAMP1 and LC3II expression and decreased Pmel17 expression was also observed in 900 μM rhododendrol-treated melanocytes (Fig. 1D). Together, these results demonstrate that exposure of melanocytes to rhododendrol inhibits melanin production and also enhances the autophagy-lysosome pathway.

By electron microscopy examinations, the number of stage IV melanosomes in the cells' cytoplasm markedly decreased, lysosome numbers also increased dramatically after rhododendrol treatment, and double-membrane enclosed autophagic vacuoles were observed to contain materials resembling stage II–III melanosomes (Fig. 2A). These results suggest that rhododendrol inhibits melanogenesis and induces subsequent autophagy-lysosome activation and autophagic melanosome degradation. To investigate the exact role of autophagy-lysosome activation in rhododendrol-treated melanocytes, we examined the effect of rhododendrol on autophagy-deficient melanocytes and autophagy-enhanced melanocytes. Cultured human melanocytes were pretreated with bafilomycin A1 or rapamycin for 3 h to inhibit or induce autophagy, respectively. Autophagy deficiency (bafilomycin A1 pretreated) significantly exacerbated rhododendrol cytotoxicity in cultured melanocytes, and this cytotoxicity was clearly abolished in melanocytes that were pretreated with rapamycin (Fig. 2B). These results suggest that autophagy induction significantly attenuates rhododendrol-induced melanocyte death. Protein expression levels of p62 and BiP were observed strikingly up-regulated by
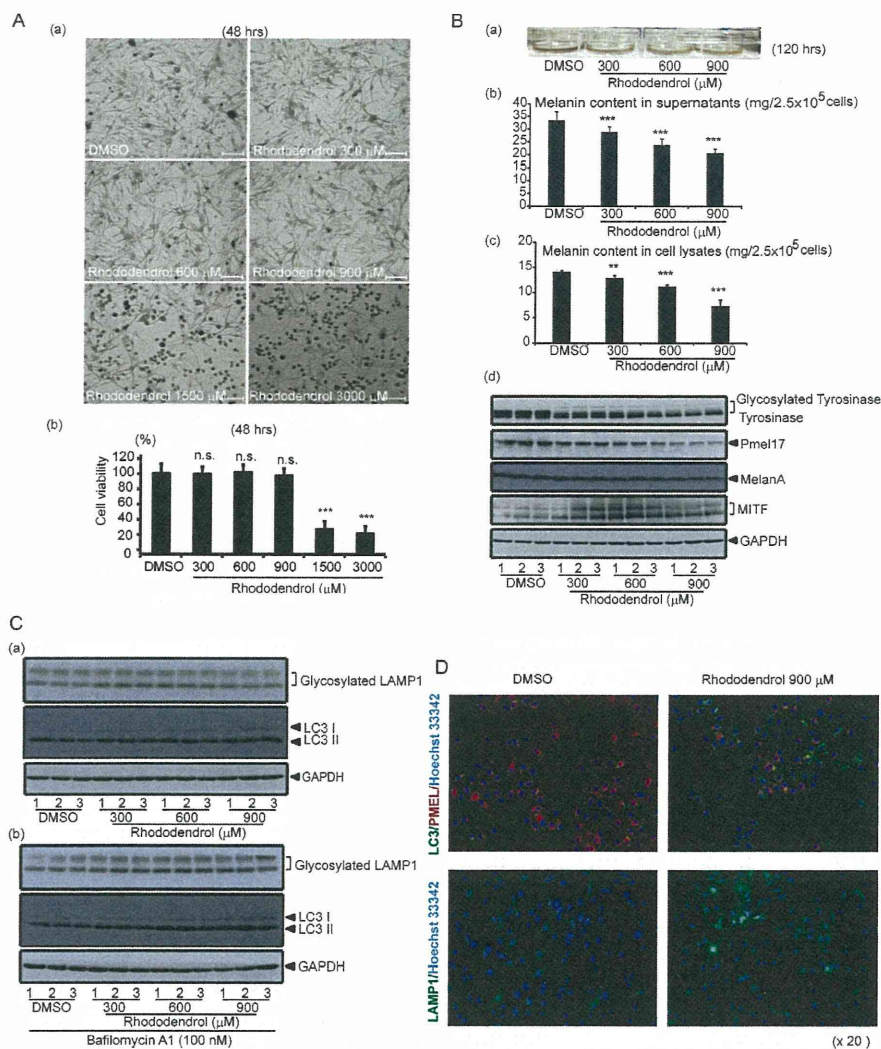
Fig. 1. Cultured human epidermal melanocytes were observed under phase contrast microscopy (A (a)), assessed by MTT assay (A (b)), cultured cells with medium were photographed (B (a)), melanin content both in culture medium (B (b)) and cell lysates (B (c)) were quantified with a Melanin Content Assay. Three days after rhododendrol treatment at the indicated concentrations, protein expressions in cell lysates were analyzed by western blotting ((B (d), C (a, b)). Melanocytes exposed to 900 μM rhododendrol were stained with the indicated antibodies and observed by confocal fluorescence microscopy (D).

rhododendrol treatment (Fig. S2). The p62 protein is known to mediate degradation of ubiquitinated proteins via the autophagosome-lysosome pathway [7], and BiP, a member of the HSP70 family found in the ER, is a marker of ER stress [8]. These results suggest that rhododendrol induced ubiquitin accumulation and ER stress in melanocytes. These findings also suggest that rhododendrol-induced ER stress might be one cause of reduced cell viability in rhododendrol-treated melanocytes.

Our results indicate that the mechanisms of rhododendrol cytotoxicity in melanocytes is more complicated than its merely acting as a tyrosinase analog that competitively inhibits normal tyrosinase activity, the rate-limiting enzyme for melanogenesis. A possible tyrosinase-mediated mechanism is that rhododendrol reaction with tyrosinase generates reactive semi-quinone free radicals that induce cellular stress and cytotoxicity [9,10], and subsequently activates the autophagy-lysosomal pathway.

Simultaneously, rhododendrol inhibits cellular melanogenesis by binding tyrosinase and by inducing autophagic melanosome degradation. However, cytotoxicity was only induced at higher rhododendrol concentrations or in autophagy-defective cells, as the autophagy-lysosome system becomes overloaded and cellular stress results from rhododendrol-originating quinones (Fig. 2C). This mechanism appeared to be specific as no cytotoxicity and no activation of autophagy-lysosome pathway were observed in cultured human primary keratinocytes or fibroblasts (data not shown). These data suggest that an impaired or dysfunctional autophagy-lysosome pathway in patients' melanocytes might be one explanation of why all consumers and all treated skin areas do not develop white blotching. Our findings suggest a possible role of autophagy-lysosome pathway in rhododendrol-induced depigmentation disorder, and individual variation in autophagic function may determine rhododendrol-application outcomes. These results expand our
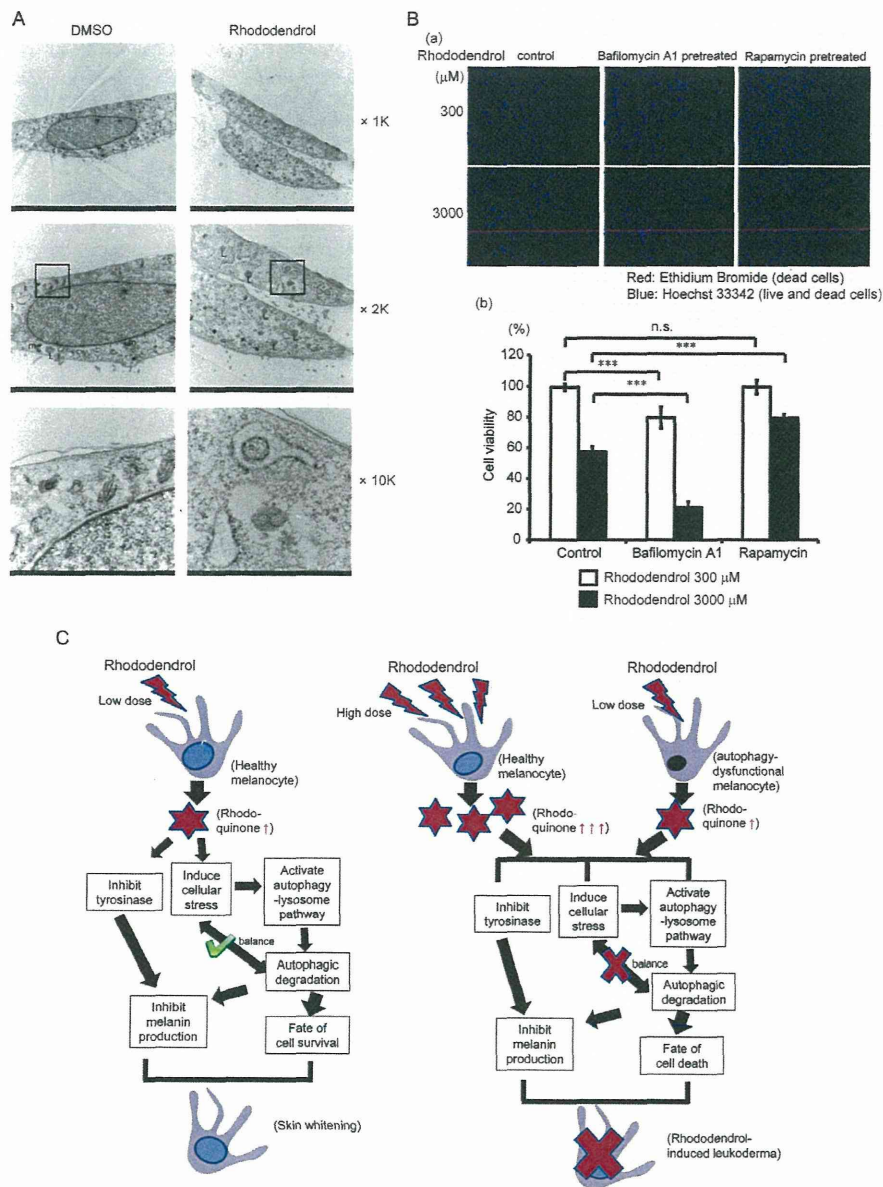
Fig. 2. Cultured human epidermal melanocytes exposed to 900 μM rhododendrol or DMSO for 72 h followed by bafilomycin A1 treatment for an additional 3 h, and then observed by electron microscopy (A). m: melanosome; L: lysosome. Cultured human epidermal melanocytes were pretreated with 100 nM bafilomycin A1 or 100 nM rapamycin for 3 h, followed by rhododendrol treatment for 72 h at the indicated concentrations. Cell staining with ethidium bromide (red) and Hoechst 33342 (blue) was observed using confocal fluorescence microscope (B (a)). After these treatments, cell viability was also evaluated by MTT assay (B). Schematic illustration of the possible mechanisms of rhododendrol-induced leukoderma (C).

understanding of the mechanisms underlying the rhododendrol-induced leukoderma.

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.2015.01.006.

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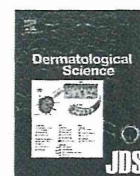[1]These authors contributed equally to this study.

Letter to the Editor

An immune pathological and ultrastructural skin analysis for rhododenol-induced leukoderma patients

A B S T R A C T

As reported in the mass media on July 2013, numerous consumers who had used the cosmetic ingredient containing rhododendrol (4-(4-hydroxyphenyl)-2-butanol, Trade name; rhododenol), which is a melanin inhibitor isolated from Acer nikoense Maxim, released from Kanebo Cosmetics Inc. (Tokyo, Japan) noticed leukoderma patches on their face, neck and hands. We have experienced 32 cases that developed leukoderma after using such cosmetics so far and skin biopsy samples in some cases were obtained from both leukoderma and pigmented lesions. A histopathological analysis for skin lesions obtained from such patients notably showed basal hypo-pigmentation, melanin incontinence, and remaining melanocytes in most patients which is not relevant in vitiligo vulgaris. Subsequently, we comprehensively carried out immunohistochemical analyses of immune-competent cells infiltration to assess the effect of the cellular immune response to inducible hypopigmentation. Furthermore, detailed morphological observations performed by electron-microscopy notably showed the presence of melanocytes with only a small number of melanosomes, dermal fibroblasts containing melanosome globules and melanophages whereas no damage associated with melanosome transfer and the basal layer apparatus. These findings provide a cue to diagnose as rhododenol-induced leukoderma differentiate from vitiligo vulgaris and for rhododendrol to induce local immunity in addition to melanocyte damage.

Rhododenol-induced leukoderma was found to have occurred in approximately 2% of the consumers who had used the cosmetics containing rhododendrol and the total number of such patients is estimated to be more than 9000 individuals. Since the products in question produced by the Kanebo corporation had been sold on the Asian market in not only Japan, but also in Korea and Taiwan, the associated health hazard is thus considered to be a serious and widespread problem. Recent research has unveiled the biochemical and physiological mechanism of melanocyte cell damage induced by rhododenol-metabolites after tyrosinase reaction [1,2]. Soon after rhododendrol was oxidated by endogeneous tyrosinase in cytoplasm, the metabolite form could thus become toxic to melanocytes due to endoplasmic reticulum and oxidative stress production [2] in addition to NO production [3], however, it is not yet investigated whether lesional immune reaction could affect the pathogeny of rhdodenol-induced leukoderma. Previously, we analyzed histopathological alteration and the infiltration of immune-competent cells in vitiligo skin followed by an investigation of the local immune milieu on melanocyte dysfunction and disappearance in the occurrence and maintenance of vitiligo vulgaris [4–7]. In this communication, we obtained hypopigmented skin specimens from patients and carried out an immune-pathological analysis of 32 lesions and an ultrastructural analysis of 6 lesions to assess morphological change of skin component cells and local cellular immune reaction. Comprehensive infiltrating cells number was summarized in Table 1. A small number of melanosomes were found remaining in the basal layer while many

were present in the dermis, along with melanin incontinentia (Fig. 1a). Lymphocytes were found invading with a focus on the upper dermis in rhododenol-induced leukoderma lesions and the mean number of CD4^{+} T cells was significantly more abundant compared to that of nonsegmental vitiligo lesions (Fig. 1b). Although the MITF or MelanA-positive melanocyte count had significantly declined compared to normal skin, complete disappearance was observed in only 4 among 31 cases (Fig. 1c). Although recent literatures implicated the toxicity of rhododendrol metabolite to melanocytes in vitro, we unexpectedly observed little structural damage of lesional remaining melanocytes (Fig. 1d). Significant number of mature melanosomes was arranged on the periphery and no vacuolization and no disturbance of melanin transfer were detected [8], considering elimination of severely affected melanocytes by phagocytosis (Fig. 1d). The basement membrane was observed under an electron microscope in order to clarify the cause of melanin dripping; although destruction of the basement membrane and hemidesmosome structure was not evident, spindle dermal fibroblasts comprising intracellular melanosome corpuscles were identified (Fig. 1e), and S-100 positive melanophages were also found invading at a distance from the basal membrane on the upper dermis (data not shown). The infiltration of fibroblasts containing melanosome complex beneath a basal membrane [9] was observed under an electron microscope in 2 cases out of 6 cases, a finding potentially indicative of rhododenol-induced leukoderma (Fig. 1e).

At the same time, prominent cellular infiltration by the invading inflammatory cells was observed focusing on the hair

Table 1

Results of an immunohistochemical analysis for immune competent cell infiltration. The data represent the mean number of infiltrating cells calculated by three independent physicians under ×100 magnification. A statistical analysis for comparison purposes was performed by using the unpaired t-test and the p-value was calculated in comparison with that obtained from normal skin.

	MelanA	S100^{+} MITF^{+}	CD3	CD8	CD4	CD4^{+} IL17A^{+}	Foxp3^{+}	CD20	CD56
Rhodo-induced leukoderma ($n=32$)	9.5 ± 9.0[**]	5.0 ± 5.4[**]	68.8 ± 39.7[*]	32.7 ± 19.2[*]	41.1 ± 17.5[**]	8.3 ± 5.7[*]	17.3 ± 10.1[*]	1.5 ± 2.3	1.5 ± 3.3
Nonsegmental vitiligo vulgaris ($n=6$)	1.7 ± 3.5[**]	1.2 ± 1.7[**]	58.2 ± 27.9[*]	39.5 ± 22.7[**]	19.7 ± 5.4[*]	8.9 ± 4.7[*]	8.6 ± 4.1	3.7 ± 3.2	4.5 ± 1.2
Normal ($n=3$)	39.3 ± 3.1	32.1 ± 5.9	15.2 ± 2.5	7.8 ± 2.4	12.3 ± 2.1	8.5 ± 1.0	7.9 ± 3.2	5.88 ± 1.1	0

[*] $p < 0.01$.
[**] $p < 0.05$.

follicle in the excised hair follicle sample, with the number of inflammatory cells therein greater than those observed in nonsegmental vitiligo even in progressive status) (Table 1). The CD4 and CD8^{+} T cell counts were substantially the same and the invasion was not CD8^{+} cytotoxic T-cell dominant, as observed in nonsegmental vitiligo. Although not only direct melanocyte damage but also the induction of immunity specific for melanocyte-associated antigens by rhododendrol were predicted, in
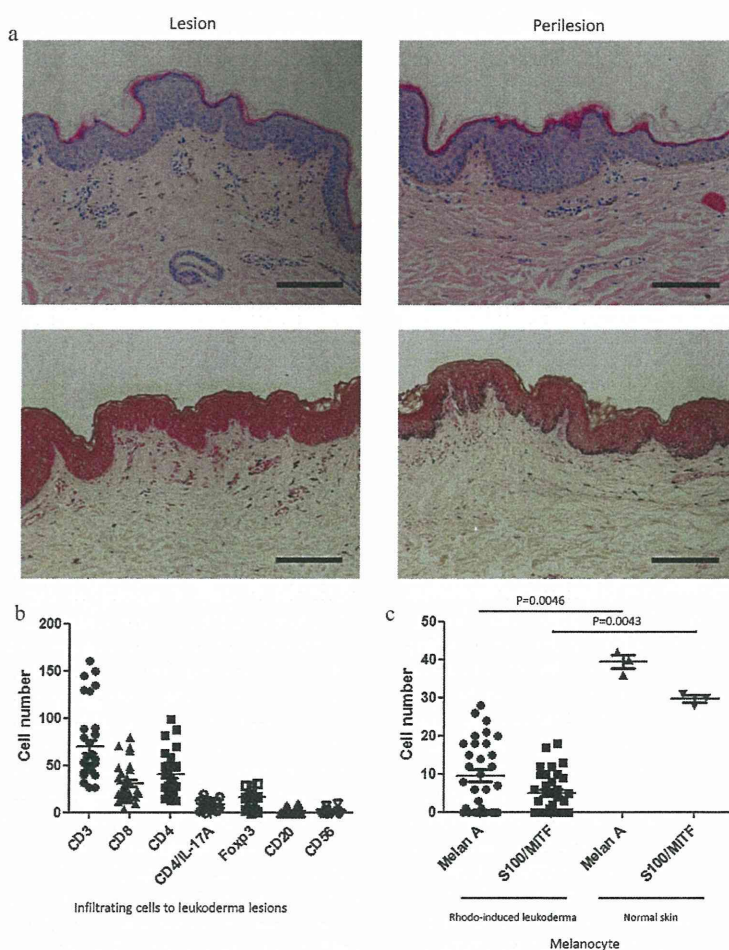
Fig. 1. (a) Histological findings of rhododenol-induced leukoderma skin specimens. The upper and lower rows show H and E staining and Masson–Fontana staining, respectively. Bar indicates 100 μm. (b) Dot plot of the immune-competent cells' number infiltrated into rhododenol-induced leukoderma lesions. Notably, T cell infiltration was predominant rather than the infiltration of NK cells and B cells. (c) Dot plot analysis for the number of melanocytes remaining on rhododenol-induced leukoderma lesions. While the number of remaining melanocytes varied between each patient, only a few cases lost MelanA[+] or MITF[+] melanocytes. (d) An ultrastructural feature for melanocyte remaining on leukoderma lesion. Visible melanosomes were mostly in stage IV and appeared to be regularly arranged along with cell membrane (left). Intracellular organelles including mitochondria and rough endoplasmic reticulum were not notably altered. Left and right panels indicate ×10,000 and ×20,000 original magnification, respectively. (e) An ultrastructural analysis for dermal fibroblasts which phagocyte melanosomes in various stages. A dermal fibroblast containing melanosomes was detected just below the basal membrane (upper left). The melanosomes were located in the intracellular lysosome, thus indicating phagocyte and enzymatic digestion (upper right). A higher magnification view shows no alteration of hemidesmosomes and the basal membrane (lower left). Upper left, upper right, and lower left panels indicate ×2000, ×15,000, and ×6000 original magnification, respectively.
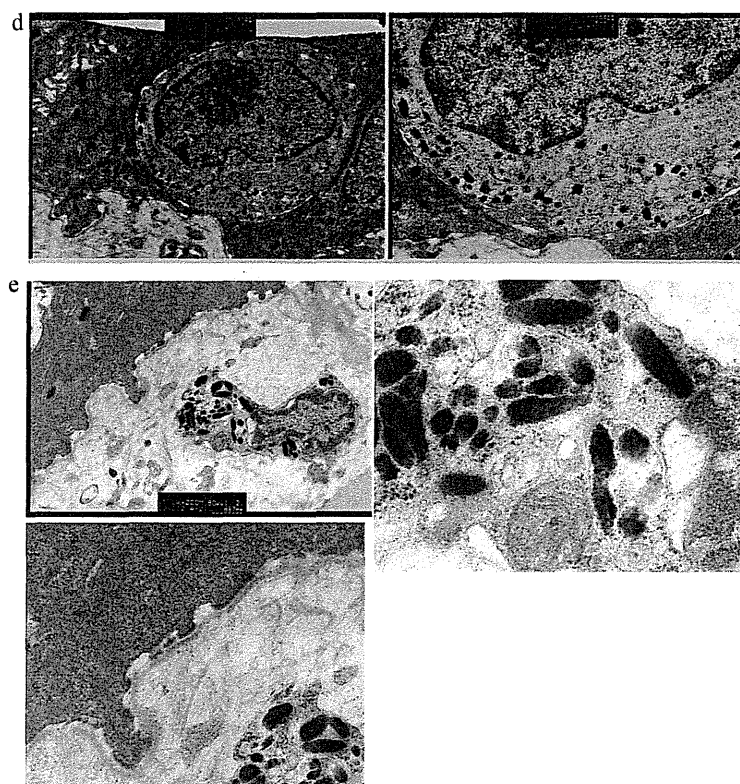
Fig. 1. (Continued).

reality, a change in the inflammatory tissue of T cells accompanying more general cytokine production may have been mainly caused. Moreover, it was suggested that the function of restricting the autoimmune reaction was maintained more than the prominent invasion of Foxp3$^+$ regulatory T cells, potentially being a result that reflects the nonspecific inflammatory response directed to all T cells axis. NK cells and B cells were not found substantially invading, in the same manner as in vitiligo vulgaris [7]. Upon observation by electron microscope in this study, melanocytes with reduced number of mature melanosomes were observed in small quantities and edema was observed between epidermal cells; however, no apparent damage to the epidermal cells was observed. The skin biopsy in this study involved the observation of skin lesions which were acquired within 2 months following discontinuing the use of the rhododenol-containing ingredient; however, no apparent damage to the melanocytes predicted from past reports was observed by electron microscope, and the image may have been observed following elimination due to apoptosis, necrosis, etc. From the observation of skin lesions under light microscopy and an electron microscope in this study, it was clarified that inflammation focusing on T cells was induced, and it is believed that future experiments capable of mimicking in vitro or in vivo conditions is warranted in order to determine whether or not this reaction was a secondary phenomenon.

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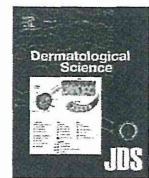
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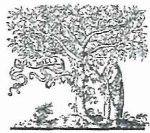
Letter to the Editor

Possible involvement of CCR4+CD8+ T cells and elevated plasma CCL22 and CCL17 in patients with Rhododenol-induced leukoderma

Rhododendrol (4-(4-hydroxyphenyl)-2-butanol), brand name: Rhododenol) is a naturally occurring phenol used in skin-whitening cosmetics and has been reported to cause a hypopigmentary disorder called Rhododenol-induced leukoderma (RIL) which developed in approximately 16,000 (2%) consumers [1]. Recent pathological analyses of RIL have revealed a direct cytotoxic effect of rhododendrol on melanocytes, which may explain the loss of melanocytes in the lesional skin [1]. In addition, we speculated that local infiltration of various immune cells may be involved in the pathophysiology of RIL [2]. A skin homing receptor C-C chemokine receptor type 4 (CCR4) is reported to be expressed in a greater proportion of CD8+ T cells in the blood of nonsegmental vitiligo patients than that observed in healthy individuals [3]. This receptor is required for skin homing of T cells via its ligands, C-C chemokine ligand (CCL) 17 [4] and CCL22 [5]. Although CCR4 is primarily expressed on CD4+ T cells and regarded as a marker of type 2 helper T cells [6], CD8+ T cells also express CCR4. These CCR4+CD8+ T cells function to secrete multiple cytokines rather than act as cytotoxic cells [7]. We herein investigated the frequencies of CCR4+ T cells and its ligands in the blood and lesional skin of RIL patients.

Nineteen patients who gave written informed consent were informed in Table S1. Percentage of CCR4-expressing cells among CD3+ T cells was 27.40% in the RIL patients and 28.20% in the nonsegmental vitiligo patients in contrast to 18.55% in healthy controls (RIL, $P = 0.0282$; nonsegmental vitiligo, $P = 0.0500$; Fig. 1A) while CCR4+CD4+ T cells was not increased in the patients. Interestingly, CCR4+CD8+ T cells were significantly increased in the patients (RIL, $P = 0.0109$; nonsegmental vitiligo, $P = 0.0104$; Fig. 1B). The percentage of CCR4+CD8+ T cells correlated to the duration after withdrawal of rhododendrol before sampling (Fig. 1C). Therefore, we focused on CCR4+CD8+ T cells in the RIL patients for further experiment. To investigate whether the CCR4+CD8+ T cells infiltrated into the RIL skins, a double immunohistochemical analysis of CCR4 and CD8 was performed (Fig. 1D), resulting in increased number of CCR4+CD8+ T cells in the RIL tissue samples (24.25/field for the RIL lesions compared to 6.50/field for healthy control skins; $P = 0.0569$) Fig. 1E). In addition, we found significantly increased ratio of CCR4+CD8+ T cells to whole CD8+ T cells in the RIL samples (39.43% for the RIL lesions compared to 8.576% for the nonsegmental vitiligo lesions and 6.280% for healthy skin samples; nonsegmental vitiligo, $P = 0.0498$; normal control, $P = 0.0144$; Fig. 1F). Peripheral blood T cells from ten patients analyzed twice. Most of them showed reduced CCR4 expression on CD8+ T cells over time (Fig. 1G). Regarding analysis for CCR4 ligands, CCL22 was expressed by infiltrating mononuclear cells in the lesional skin of RIL patients (Fig. 2A–C), although no CCL17 expression was observed (data not shown). Number of CCL22+ cells was increased in vitiligo and RIL lesions compared to healthy skins (Fig. 2D). The cells expressing CCL22 were positive for CD1a or CD68, suggesting dermal dendritic cells or macrophage, respectively (data not shown). The plasma CCL17 and CCL22 concentrations were elevated in the RIL patients compared to those in vitiligo and healthy individuals (Fig. 2E and F depicted CCL22 and CCL17, respectively).

As increased frequencies of CCR4+CD8+ T cells in the peripheral blood have been reported in patients with cutaneous diseases, in which immunity is regarded to be involved [3,8], we investigated the frequency of CCR4+ T cells in RIL patients in order to explore the possible involvement of immunity and potential therapeutic targets for this disease. Consequently, we found that the frequency of CCR4+CD8+ T cells was increased in the peripheral blood of patients with RIL as well as those with nonsegmental vitiligo, as reported previously by Zhang et al. [3]. Although both the RIL and vitiligo patients in this study showed increased frequencies of CCR4+CD8+ T cells, these cells are expected to target different antigens in each disorder. Pathogenic CD8+ T cells in nonsegmental vitiligo, which may express CCR4 in order to migrate into the skin, are thought to be specific for melanocyte antigens [9]. In fact, melanocyte antigen-specific CD8+ T cells are reported to express another skin homing marker, cutaneous leukocyte-associated antigen (CLA) [9]. On the other hand, in patients with RIL, considering that symptoms usually develop only at Rhododenol-applied sites [2], CCR4+CD8+ T cells may target endogenous molecules modified by rhododendrol. In addition to the specificity of skin homing T cells, further studies focusing on the functions of these CCR4+CD8+ T cells, such as cytokine secretion, are needed to elucidate what role CCR4+CD8+ T cells play in the pathogenesis of these diseases. Considering that CCL22 is a dominant ligand in inducing receptor desensitization and internalization [5], the reduction of CCR4 expression on CD8+ T cells over time may be explained by the dominant expression of CCL22 in the lesional skin and plasma. In cases of atopic dermatitis, CCL17/TARC is considerably important with respect to the inflammatory process [10]; although both CCL17 and CCL22 in plasma were increased in the RIL patients, CCL22-assiatied chemotaxis contributing to skin inflammation may be the more characteristic of RIL. It is limitation of this study that specimens from 98% of the consumers without leukoderma were not analyzed due to ethical considerations.
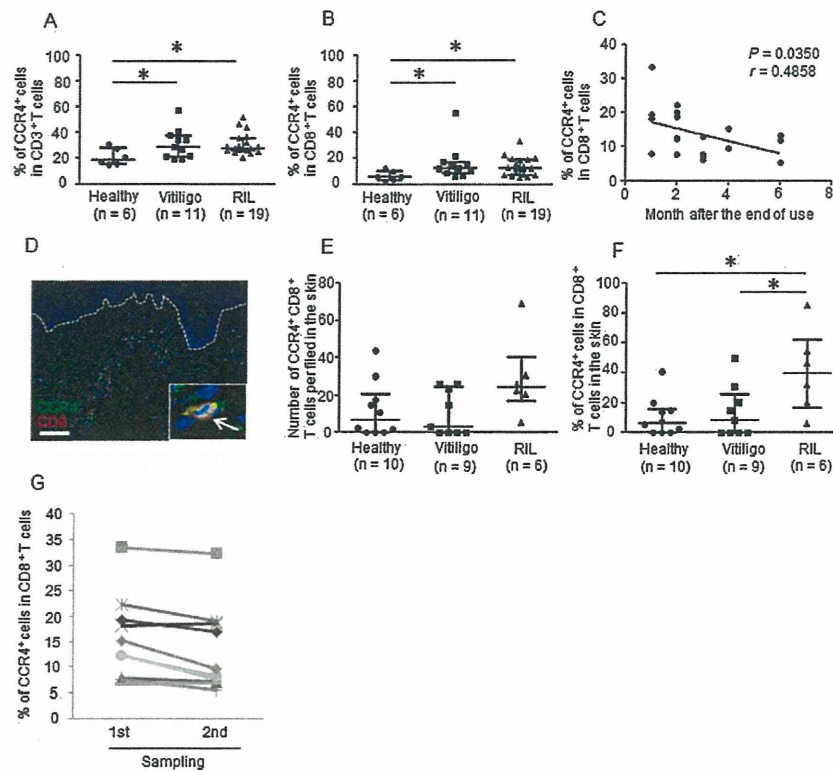
In conclusion, the frequency of CCR4+CD8+ T cells and CCL17 and CCL22 concentrations were increased in the blood of RIL

Fig. 1. Increased percentage of CCR4+ cells among CD8+ T cells in the peripheral blood and skin of Rhododenol-induced leukoderma patients. The percentages of CCR4+ T cells among (A) CD3+ T cells, (B) CD8+ T cells in the PBMCs of healthy control, nonsegmental vitiligo and RIL samples are shown. (C) Correlation between percentage of CCR4+ cells in CD8+ cells and month after the withdrawal of Rhododenol-containing cosmetics. (D) Representative double immunostaining for CCR4 (green) and CD8 (red) in the RIL skin. Inbox shows double positive cell with higher magnification. Bar indicates 100 μm. (E, F) Infiltration of CCR4+CD8+ T cells into the skin of the healthy control, nonsegmental vitiligo and RIL samples. Bars represent the median ± interquartile range. *P ≤ 0.05. (G) Percentage of CCR4+ cells in CD8+ cells were compared in ten RIL patients.
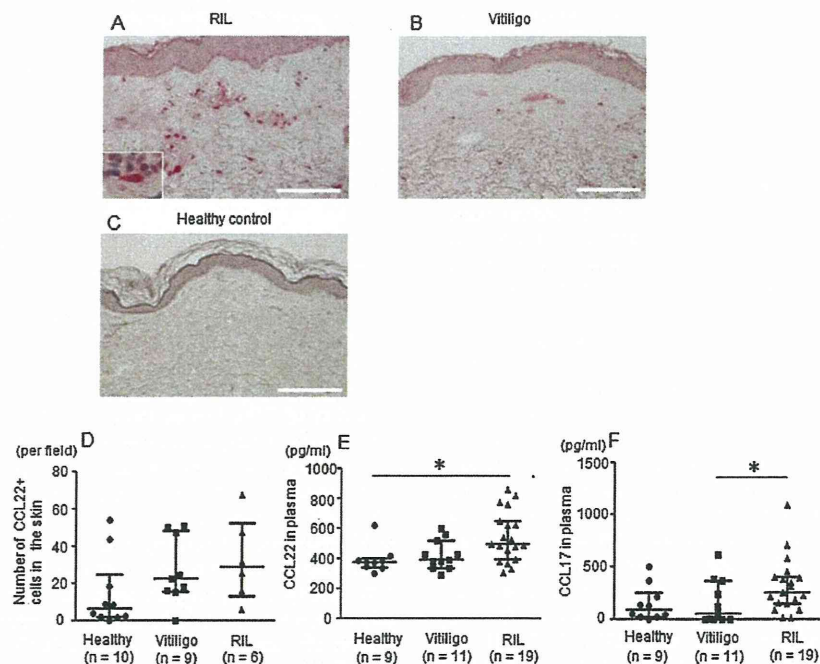
Fig. 2. Increase in CCL17 and CCL22 in the plasma and skin of the Rhododenol-induced leukoderma patients. Representative immunostaining for CCL22 (red, higher magnification view shown in box) in the lesional skin of RIL patient (A), nonsegmental vitiligo patient (B), and healthy control (C). Bar indicates 200 μm. (D) Comparison of numbers of CCL22+ cells in the skin sample among RIL, nonsegmental vitiligo, and healthy control. Comparison of concentration of plasma CCL22 (E) and CCL17 (F) among RIL, nonsegmental vitiligo, and healthy control. Bars represent the median ± interquartile range. *P ≤ 0.05.