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Effects of rhododendrol and its metabolic products on melanocytic cell growth

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ABSTRACT

Background: Rhododendrol (RD), a skin-whitening agent, is believed to be associated with cases of cosmetics-related leukoderma that have been reported in Japan. Recently, we have shown that RD is catalyzed by tyrosinase to produce putative toxic metabolites RD-catechol and RD-cyclic catechol.
Objective: To examine the cytotoxicity and production of reactive oxygen species (ROS) in melanocytic cells by RD and its metabolic products.
Methods: The growth inhibitory effect of RD or its metabolite on the normal human epidermal melanocyte (NHEM) and B16F1 cells was assessed by cell counting or WST assay. ROS production was detected by flow cytometry and confocal microscopy after cells were treated with 2′,7′-dichlorofluorescein and RD or its metabolite.
Results: Growth of NHEM derived from African American (NHEMb) and B16F1 cells was suppressed by 300 μM or more RD. Growth inhibitory activity of RD (IC50 of B16F1: 671 μM) was weaker than hydroquinone (IC50 of B16F1: 28.3 μM) or resveratrol (IC50 of B16F1: 27.1 μM). Flow cytometric analysis detected ROS production in the NHEMb and B16F1 cells exposed to RD. However, neither RD nor H_2O_2 increased the subG1 fraction of these melanocytic cells. RD-catechol and RD-cyclic catechol inhibited growth of NHEMb and B16F1 cells much more strongly than did RD. RD-catechol, as well as RD, produced ROS detected by both flow cytometry and immunostaining, while RD-cyclic catechol produced a hardly detectable amount of ROS in B16F1 cells.
Conclusions: These results suggest that RD exerts the cytotoxicity in melanocytic cells through its oxidative metabolites and that ROS plays a role in RD-mediated cytotoxicity.
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1. Introduction

Melanin pigment is produced in the melanocytes and transferred to the surrounding epidermal keratinocytes to protect genomic DNA from ultraviolet light [1]. Overproduction and accumulation of melanin pigment in the epidermis and/or dermis causes cutaneous hyperpigmentation such as freckles, melasma, senile lentigines and acquired bilateral dermal melanocytosis. Thus, the inhibition of melanogenesis has been the focus of medical treatment and cosmetics especially on the sun-exposed areas. Melanogenesis is regulated by the key enzyme, tyrosinase, which is essential for the first step of melanin biosynthesis, the conversion of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and subsequent oxidation to dopaquinone [2]. Dopaquinone is a highly reactive intermediate and gives rise to eumelanin production with the participation of tyrosinase-related protein-1 (TYRP-1) and TYRP-2 [3,4], while intervention of L-cysteine switches to pheomelanin production [2,5]. Pheomelanin has been reported to be cytotoxic due to the production of reactive oxygen species (ROS) [6–8]. Thus, suppression of excess amounts of melanin and suppression of intracellular ROS is necessary biochemical abilities of safe and ideal skin-whitening agent.

More than ten kinds of skin-whitening compounds have been produced and used as cosmetic ingredients. These quasi-drugs prevent or improve hyperpigmentation by inhibiting tyrosinase activity (ascorbic acid, kojic acid, arbutin and ellagic acid) or decreasing tyrosinase protein (linoleic acid) [9]. They have been used as cosmetic ingredients without causing the prominent

Abbreviations: RD, rhododendrol; NHEM, normal human epidermal melanocytes; ROS, reactive oxygen species; DOPA, dihydroxyphenylalanine.

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leukoderma on the application sites. The 4-*tertiary*-butylphenol (4-TBP) is known to be the most potent phenolic derivative causing occupational leukoderma [10–12] by inducing apoptosis in melanocytes [13]. Monobenzyl ether of hydroquinone (MBEH) is the only drug that the Food and Drug Administration has approved for depigmentation therapy in the United States. Unlike 4-TBP, MBEH induces necrotic cell death in melanocytes without activating the caspase cascade or DNA fragmentation [14].

RS-4-(4-hydroxyphenyl)-2-butanol (rhododendrol or rhododenol, RD) had been used since 2011 as a skin-whitening agent of cosmetics produced and sold by Kanebo, a cosmetics company in Tokyo, Japan. In July 2013, the cosmetics containing RD were recalled by the company because approximately 2% of users developed depigmentation on their face, neck and/or hands. The Special Committee on the Safety of Cosmetics Containing Rhododenol, established by the Japanese Dermatological Association on July 17, 2013, labeled this cosmetics-associated depigmentation as RD-induced leukoderma [15]. Clinical characteristics of RD-induced leukoderma show depigmentation of varying severity that develops after the use of cosmetics mainly at the site of use. In most cases repigmentation of a part or all the affected areas is evident after discontinuation [15].

Until recently it was considered that depigmentation by RD-containing cosmetics resulted from competitive inhibition of tyrosinase by RD. However, approximately 20% of the patients are still suffering from leukoderma on the face, neck and/or hands without repigmentation. In addition, biopsied samples taken from depigmented lesions of RD-induced leukoderma contain few or no detectable epidermal melanocytes [15]. These clinicopathological observations suggest that RD exhibits a significant cytotoxicity to the epidermal melanocytes by producing cytotoxic metabolites through the tyrosinase-catalyzed oxidation. Tyrosinase is able to oxidize, in addition to L-tyrosine, a number of phenols and catechols to form *ortho*-quinones [16]. Moreover, Sasaki et al. has reported that RD mediates its cytotoxicity in a tyrosinase-dependent manner, since suppression of tyrosinase results in the depressed cytotoxicity of RD in melanocytes [17,18]. Recently we have reported that RD was incorporated into the tyrosinase-dependent biochemical pathway and produced RD-catechol and RD-cyclic catechol in addition to the corresponding *ortho*-quinones [19,20]. Moreover, RD-quinone was bound to non-protein thiols and proteins in B16F1 cells through cysteinyl residues [21].

In order to elucidate further the molecular mechanism of cytotoxicity of RD in melanocytic cells, we have analyzed the effects of RD and its metabolic products RD-catechol and RD-cyclic catechol on the growth of melanocytic cells and ROS production in them. RD-catechol and RD-cyclic catechol showed cytotoxicity much stronger than RD and RD-catechol as well as RD generated ROS in B16F1 cells. The results obtained suggest that RD exerts its cytotoxic effect through its metabolic products and that the ROS production is related to RD-associated cytotoxicity.

2. Materials and methods

2.1. Cells and cultures

Normal human epidermal melanocytes (NHEMa, Asian/Caucasian, newborn, Kurabo Cat KM-4009, Strain No. 00378) were purchased from Kurabo Industries Ltd., Japan and cultured in the medium DermaLife® BM (LKB-LM0004) supplemented with DermaLife®M LifeFactors (LMK-LS1041). Pigmented human epidermal melanocytes derived from an African American female (NHEMb, CC-2586, Lot 237204) were purchased from Lonza Walkersville Inc. (MD) and cultured in the medium MBM-4 BulletKit™ supplemented with endothelin-3. Mouse melanoma B16F1 cells were cultured in Dulbecco's modified Eagle medium supplemented by 10% fetal bovine serum and antibiotics.

2.2. Assay for cytotoxicity of phenolic compounds

To examine the cytotoxicity of phenolic compounds to NHEM and B16F1 cells, cells were seeded at 1.5×10^5/6 cm dish and cultured for 48 h. Cells were then refed with media containing 0–800 μM RD and cultured for five or seven days. Numbers of

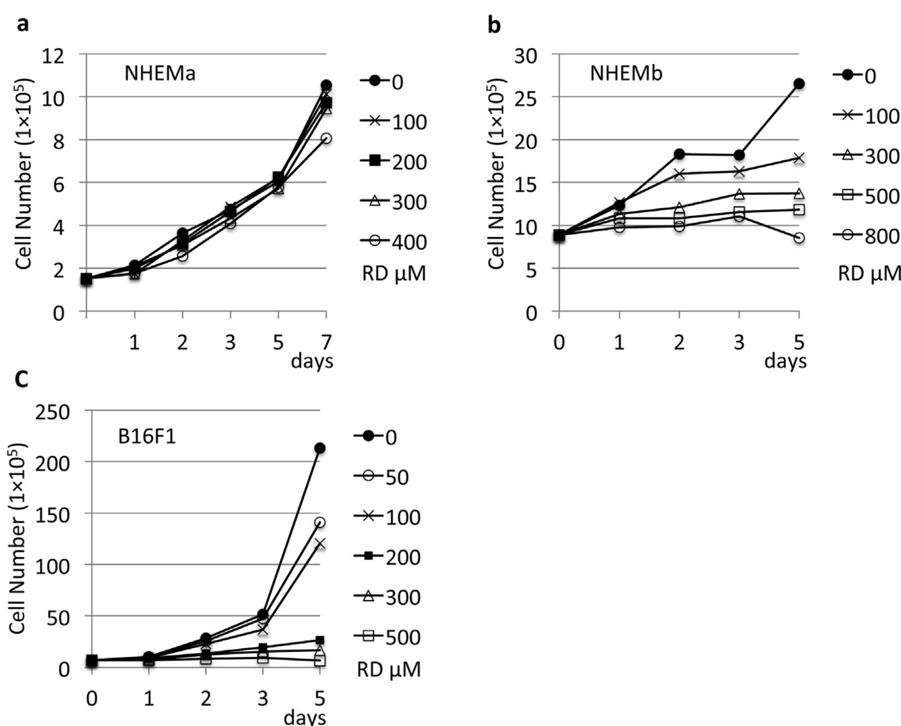

Fig. 1. RD suppressed growth of NHEMb and B16F1 cells but not that of NHEMa. Cells were cultured with RD (50–800 μM) and numbers of viable cells were counted for five or seven days.

viable cells were counted using a hemocytometer. To determine IC50 of the phenolic compounds, 2000 cells/well were seeded in 96-well plates, cultured for 24 h and then refed with the medium containing RD, arbutin, hydroquinone or resveratrol for a further 72 h. Numbers of viable cells were counted by WST assay (Cell Counting Kit-8, Dojindo, Wako Pure Chemical Industries Ltd., Japan).

2.3. Chemicals

Mushroom tyrosinase and arbutin (4-hydroquinone-D-glucopyranoside) were purchased from Sigma–Aldrich (MO). L-Tyrosine, hydroquinone (1,4-dihydroxybenzene) and resveratrol (3,5,4′-trihydroxy-*trans*-stilbene) were procured from Wako Pure Chemicals Industries Ltd., Japan. Rhododendrol (*RS*-4-(4-Hydroxyphenyl)-2-butanol, RD) was kindly provided by Innovative Beauty Science Laboratory, Kanebo Cosmetics Inc., Japan.

2.4. Preparation of RD-catechol and RD-cyclic catechol

Initial preparation of these catechols on an mg scale was reported in Ref. [19]. However, we needed them in greater amounts and therefore made the following alterations to our methods. For the rhododendrol-catechol, a solution of rhododendrol (166 mg, 1.0 mmol, dissolved in 1 mL ethanol), ascorbic acid (1056 mg, 6 mmol), and Na_2HPO_4 (852 mg, 6 mmol) was prepared with 100 mL 50 mM sodium phosphate buffer (pH 5.3). The resulting pH was 5.3. The mixture was vigorously shaken at 37 °C upon which mushroom tyrosinase (58 mg; 100,000 U) in 2 mL buffer was added. After 60 min, HPLC analysis showed that most of the ascorbic acid was consumed while 7% of the substrate rhododendrol remained. Thus, the oxidation was stopped by adding 400 mg $NaBH_4$ and then 20 mL 1 M HCl. The mixture was extracted with ethyl acetate (200 mL and 100 mL), and the organic layers were combined, washed once with 40 mL saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was dissolved in 2 mL DMSO and subjected to preparative HPLC (see below) to afford, after evaporation, 138 mg (80% yield) of rhododendrol-catechol as colorless oil (HPLC purity > 99%).

For the rhododendrol-catechol, a solution of rhododendrol (166 mg, 1.0 mmol, dissolved in 1 mL ethanol) was prepared with 100 mL 50 mM sodium phosphate buffer (pH 5.3). The mixture was vigorously shaken at 37 °C upon which mushroom tyrosinase (58 mg; 100,000 U) in 2 mL buffer was added. After 20 min, HPLC analysis showed that most (95%) of the substrate rhododendrol

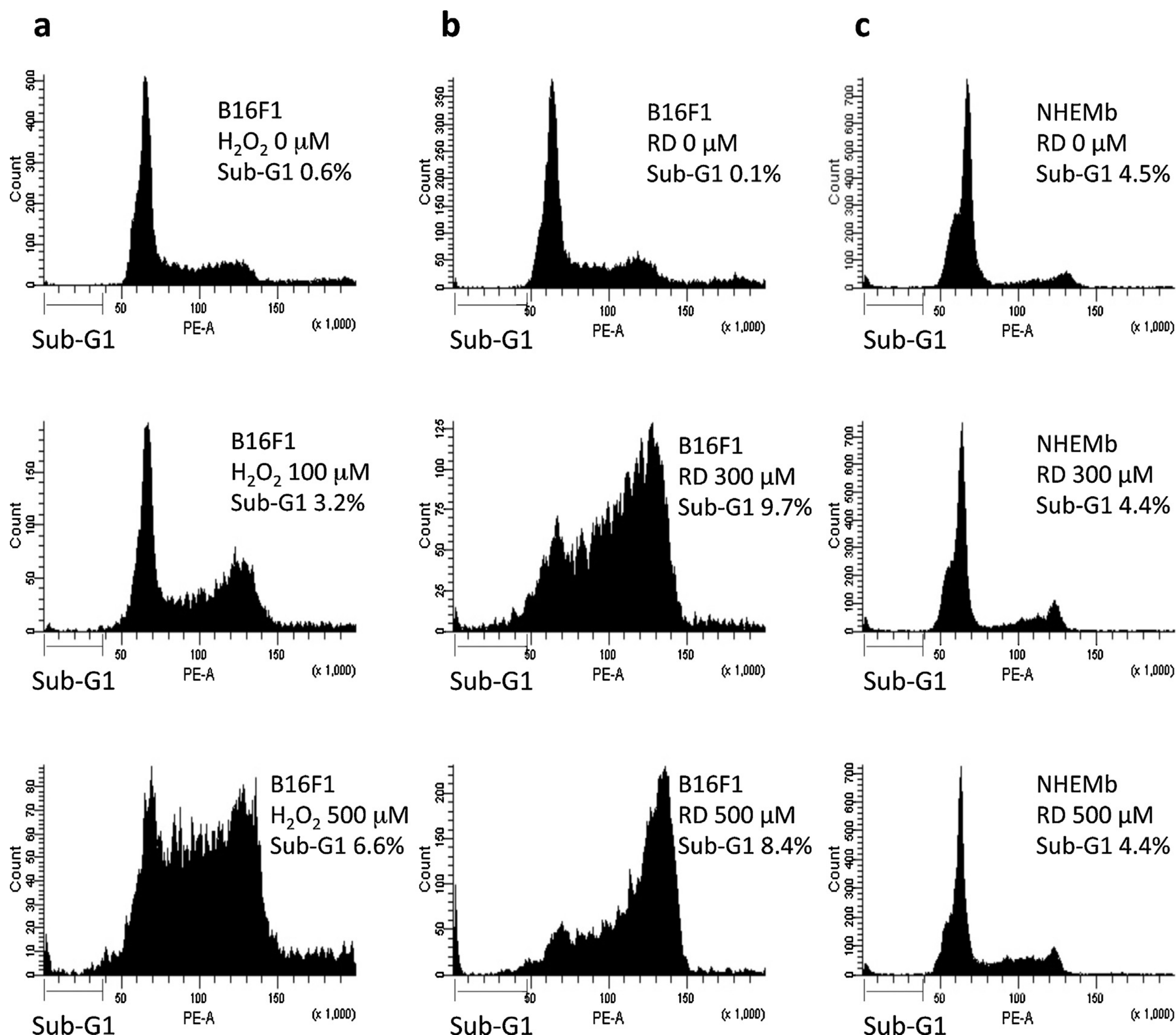

Fig. 2. RD exposure did not increase the subG1 fraction of NHEMb and B16F1 cells. After NHEMb and B16F1 cells were cultured with RD, adherent and floating cells were collected and processed for flow cytometric analysis. Population percentage of the subG1 phase is quantified and shown in the figures.

was consumed. Thus, the oxidation was stopped by adding 400 mg $NaBH_4$ and then 20 mL 1 M HCl. The mixture was extracted with ethyl acetate (200 mL and 100 mL), and the organic layers were combined, washed once with a 40 mL saturated NaCl solution, dried over anhydrous Na_2SO_4 and evaporated to dryness under reduced pressure. The residue was dissolved in 2 mL DMSO and subjected to preparative HPLC (see below) to afford, after evaporation, 32 mg (18% yield) of rhododendrol-cyclic catechol as pale yellow oil (HPLC purity 97%).

Preparative HPLC was performed as follows. An HPLC system consisted of a JASCO PU-2080 Plus pump (JASCO Co., Tokyo, Japan), a Shiseido C18 column (Capcell Pak MG; 20 × 250 mm; 5 μm particle size, Shiseido, Tokyo, Japan) and a JASCO UV/visible detector at 280 nm (JASCO Co., Tokyo, Japan). The mobile phase was 0.4 M formic acid:methanol, 60:40 (v/v) at 40 °C at a flow rate of 7.0 mL/min. RD, RD-catechol, and RD-cyclic catechol were dissolved at 100 mM concentrations in ethanol and stored at −80 °C until use.

2.5. Measurement of ROS

After cells were cultured in the medium containing 5 μM CM-H2DCFDA (DCF, Invitrogen) for 30 min at 37 °C, they were washed with PBS, refed with the medium containing RD, RD-catechol or RD-cyclic catechol and cultured for a further 24 h. Adherent and floating cells were then combined and washed with PBS twice. The cells were suspended in 600 μl PBS and processed for flow cytometry to quantify the FITC-H (Becton Dickinson, CA, USA) using the Cell QUEST program. For the positive control, the cells were cultured with H_2O_2 (100 or 500 μM) for 24 h. Adherent and floating cells were then collected and processed as described above. We also detected intracellular ROS using a confocal fluorescence microscope (ZEISS/confoCor3LSM510META). For this, 3×10^4 B16F1 cells were separately seeded on round glass coverslips that were coated with Atelo Cell IPC-30 (KOKEN, Tokyo) and put into each well of 12-well plates. After cells were cultured for 24 h, they were stained with 5 μM CM-H2DCFDA for 30 min. They were then cultured in the medium containing RD (500 μM). After being cultured for 24 h, they were incubated at room temperature in 4% paraformaldehyde containing Hoechst33342 for 15 min. By these methods, intracellular ROS and cellular nuclei were stained to green and blue, respectively.

2.6. Flow cytometric analysis

Cells were cultured in the medium containing RD, RD-catechol or RD-cyclic catechol for 48 h and processed as described previously [22]. The adherent and floating cells were combined, washed in PBS, dehydrated in 70% cold ethanol and stored on ice for 2 h. They were then rehydrated in cold PBS and incubated in the presence of RNase A (100 μg/ml) (Invitrogen, CA) at 37 °C for 30 min. After incubation, the cells were rinsed twice in cold PBS and suspended in 800 μl of PBS containing 50 μg/ml propidium iodide (Wako) at 4 °C for 2 h. SubG1, G1, S, and G2/M populations were quantified with a FACSCalibur flow cytometer (Becton Dickinson, CA) using the Cell QUEST program.

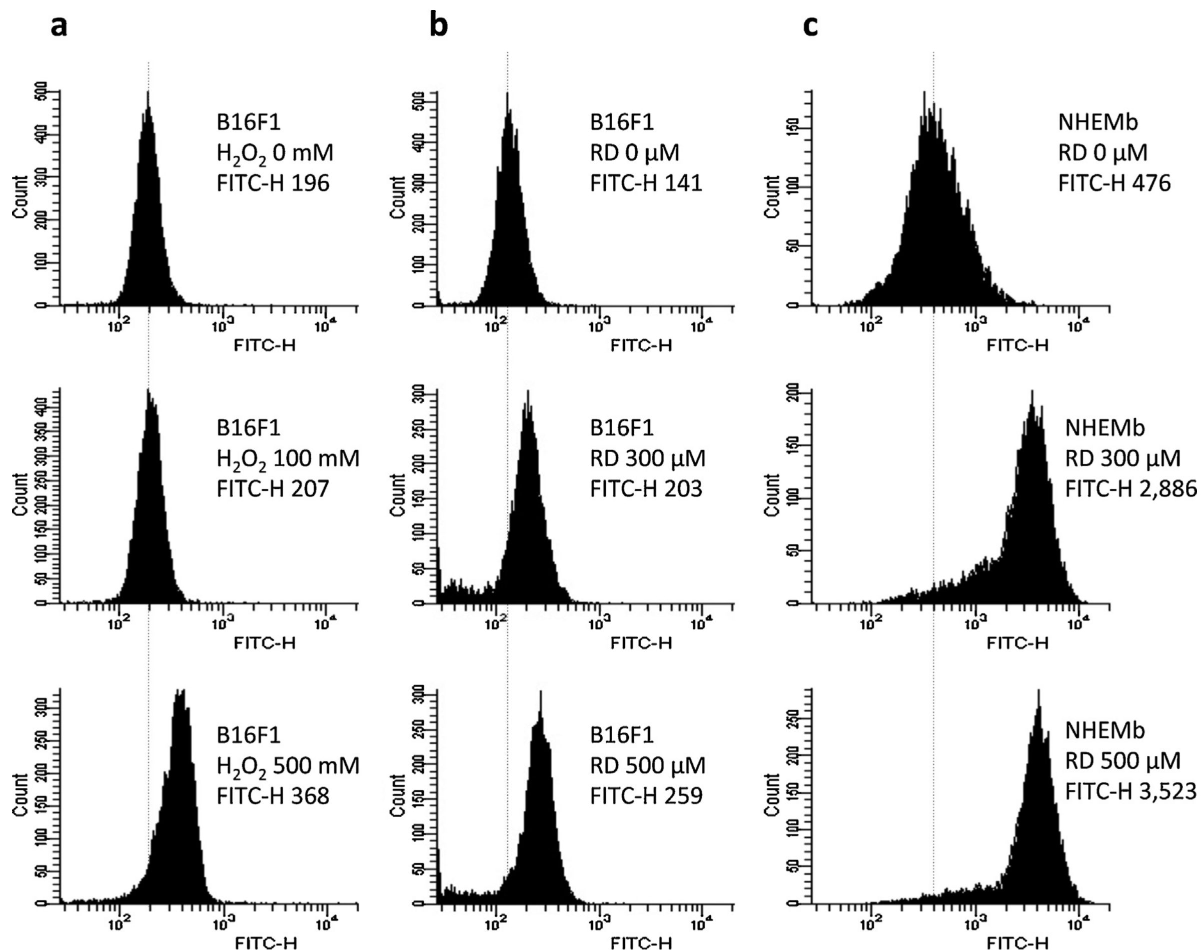

Fig. 3. RD produced ROS in NHEMb and B16F1 cells. (a) As a positive control, B16F1 cells were treated with DCF for 30 min and then cultured in the presence of H_2O_2 (100 or 500 μM) for 24 h. (b, c) After B16F1 (b) and NHEMb (c) cells were treated with DCF for 30 min, cells were cultured with RD for 24 h. ROS was detected by flow cytometry as described in Section 2.

2.7. Statistics

Mean standard error ($n = 8$) and statistical significance of data were analyzed by the Student's t-test.

3. Results

3.1. RD suppressed growth of pigmented human melanocytes and B16F1 cells

First, we examined the effect of RD on the growth of normal human epidermal melanocytes (NHEMa and NHEMb) and mouse B16F1 melanoma cells. RD did not affect the growth of NHEMa cells derived from Asian/Caucasian newborns that contained only small amounts of melanin pigments. However, RD suppressed the growth of pigmented NHEMb and B16F1 cells at the concentration of 500 μM and 200 μM, respectively (Fig. 1). To compare the cytotoxicity of RD with other phenolic compounds, we examined the growth of the melanocytic cells in the presence of arbutin, hydroquinone or resveratrol and determined their IC50. As shown in Table 1, IC50 of RD in B16F1 (671 μM) and NHEMb cells (1053 μM) was significantly higher than resveratrol (27.1 and 38.2 μM) and hydroquinone (28.3 and 9.4 μM), but much lower than arbutin (41,200 and 12,200 μM). This suggests that RD-mediated cytotoxicity was relatively moderate compared to hydroquinone and resveratrol, and arbutin has little cytotoxicity to melanocytic cells.

To analyze the molecular mechanism of growth suppression of melanocytic cells, flow cytometric analysis was performed using cells cultured for two days in the presence of RD. The NHEMb and B16F1 cells were cultured with 300 or 500 μM RD or 500 μM H_2O_2, concentrations at which almost all the cells were degraded. As shown in Fig. 2, flow cytometry did not detect the increase of the subG1 fraction (Fig. 2a–c). Instead, the results showed a remarkable increase in the S through G2/M phase, suggesting the cell cycle was arrested halfway through the process of DNA replication. Activated caspase 3 was not observed in the cells exposed to RD (data not shown). These results suggest that the major mechanism of growth suppression by RD did not occur through the process of apoptotic cell death but through the cell cycle arrest and cell degradation.

Table 1
IC50 of phenol derivatives in cultured cell.

	IC50 (μM)[a]	
	B16F1	NHEMb
RD	670.8	1052.5
Arbutin	41,200	12,200
Hydroquinone	28.3	9.4
Resveratrol	27.1	38.2

[a] IC50 is the concentration of each phenolic compounds that suppressed cell growth to 50% after cells were cultured with drugs for 72 h.

3.2. RD produces ROS in melanocytic cells

Flow cytometry was used to measure the levels of intracellular ROS through the use of cells first treated with DCF and then cultured with RD as described in Section 2. When NHEMb and B16F1 cells were exposed to DCF and RD, they produced ROS, the quantity of which was clearly detected by flow cytometry (Fig. 3). The quantity of ROS in the NHEMb exposed to 300 μM RD was larger than that of B16F1 cells exposed to 300 μM RD or 500 μM H_2O_2, suggesting that epidermis-derived melanocytes are more susceptible to ROS production by RD (Fig. 3a–c). We also detected ROS by confocal microscopy in the B16F1 and NHEMb cells after they were cultured in the RD-containing media

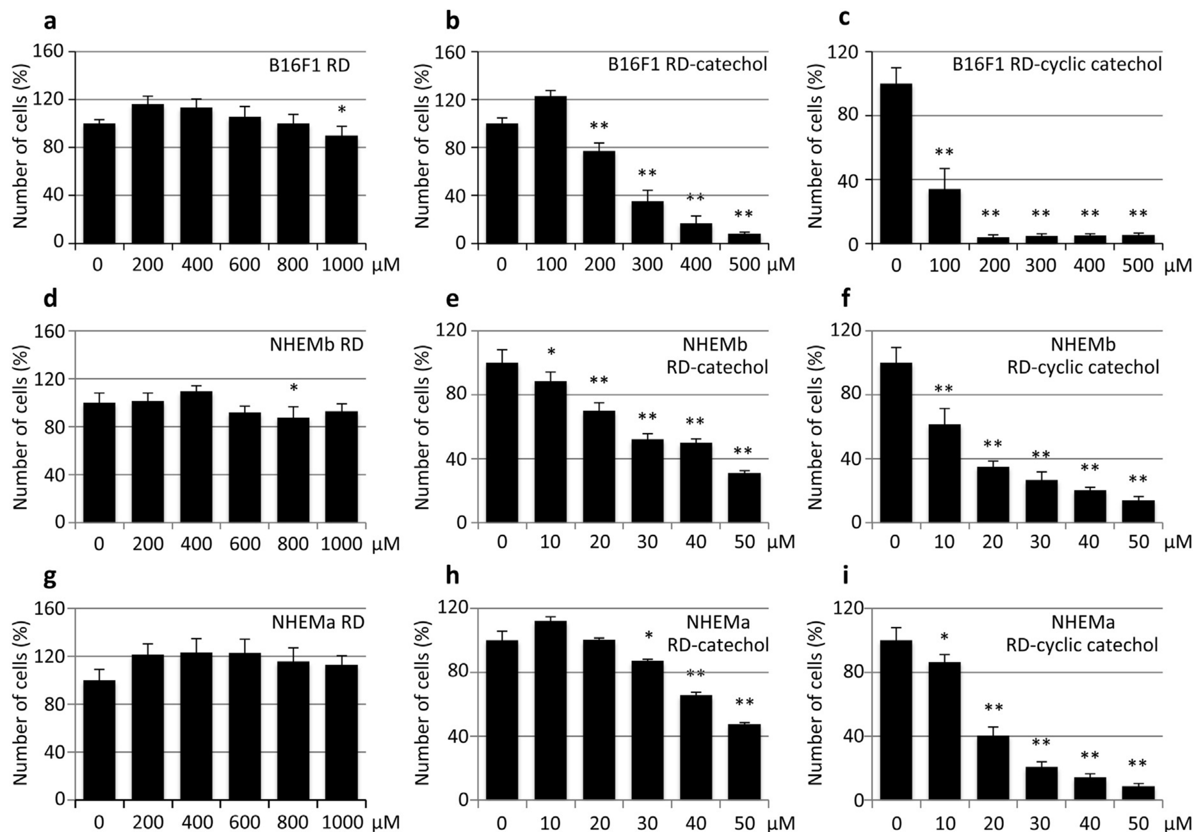

Fig. 4. RD-catechol and RD-cyclic catechol suppressed growth of B16F1 cells more significantly than RD. B16F1 (a–c), NHEMb (d–f) and NHEMa (g–i) cells were cultured with RD, RD-catechol or RD-cyclic catechol for 24 h and numbers of cells were counted by WST assay. $^{*}p < 0.05$, $^{**}p < 0.0001$ *versus* untreated control.

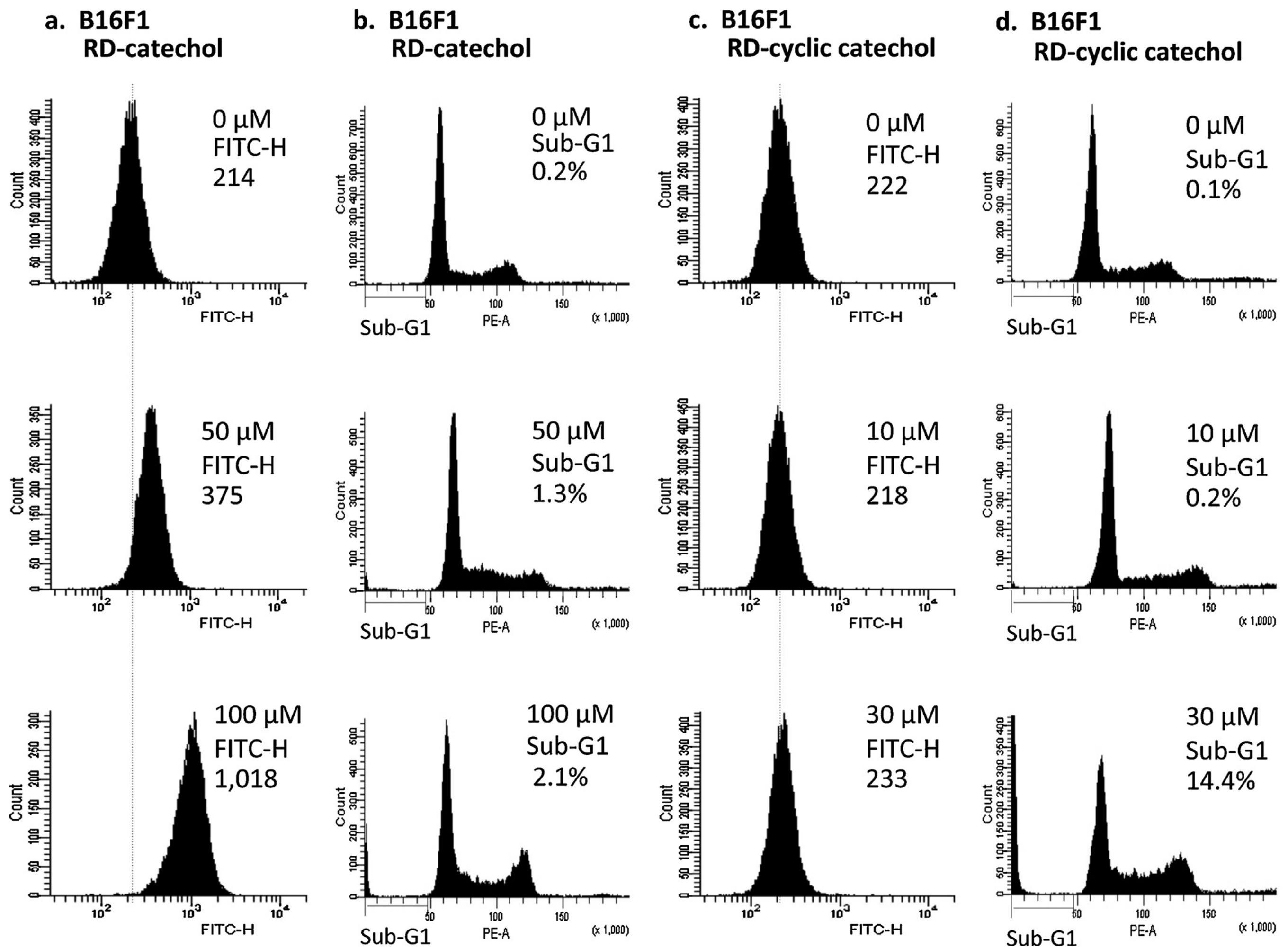

Fig. 5. ROS production and cell cycle of B16F1 cells exposed to RD-catalytic metabolites. ROS production of B16F1 cells exposed to RD-catechol (a), cell cycle analysis of B16F1 cells exposed to RD-catechol (b), ROS production of B16F1 cells exposed to RD-cyclic catechol (c) and cell cycle analysis of B16F1 cells exposed to RD-cyclic catechol (d) were analyzed by flow cytometry as described in Section 2.

(Supplementary Fig. 1). Despite a large amount of ROS being detected in the RD-treated cells, apoptotic cell death was not observed in these melanocytic cells as described above. This suggests, therefore, that ROS production did not result in the cell degradation by the process of apoptotic cell death.

Supplementary Fig. 1 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.07.010.

3.3. RD-metabolites suppressed growth of B16F1 more significantly than RD

The major products of tyrosinase-catalyzed RD oxidation were RD-catechol and RD-cyclic catechol [19]. We analyzed their inhibitory effects on the growth of B16F1 cells and production of ROS. After B16F1 cell were cultured with RD, RD-catechol or RD-cyclic catechol for 24 h, the number of cells was counted by WST assay. Survival cells were reduced in the RD-catechol- or RD-cyclic catechol-treated cells more significantly in than RD-treated cells (Fig. 4a–i). The number of survival cells was remarkably reduced in the NHEMa and NHEMb cells compared to B16F1 cells in the medium containing RD-catechol or RD-cyclic catechol at a concentration of greater than 50 or 20 μM, respectively (Fig. 4e, f, h and i).

Because B16F1 cells cultured for 48 h in the presence of >100 μM RD-catechol or >30 μM RD-cyclic catechol severely degraded, flow cytometric analysis was performed in the media containing 100 μM RD-catechol or 30 μM RD-cyclic catechol (Fig. 5). Results indicated that RD-catechol produced a significant amount of ROS in B16F1 cells, while RD-cyclic catechol did not produce a detectable amount of ROS (Fig. 5a and c). Immunocytochemistry also detected ROS production in the RD- and RD-catechol-treated B16F1 cells (Fig. 6). However, RD-cyclic catechol produced only a small amount of ROS in accordance to flow cytometric analysis. Differing from RD-treated cells, RD-catechol- and RD-cyclic catechol-treated cells showed a slightly increased subG1 fraction (Fig. 5b and d). These results suggest that RD-mediated cytotoxicity depends on the RD-catalyzed metabolites, RD-catechol and RD-cyclic catechol, but ROS production is not required for degradation of melanocytic cells by RD-cyclic catechol.

4. Discussion

Occupational leukoderma was reported on hands and fingers among the workers of Japanese raspberry factory preparing 4-(4-hydroxyphenyl)-2-butanone, the structure of which is very similar to RD [23]. The biochemical characterization of RD was first described by Akazawa et al. [24] as a phenolic compound, isolated from the bark of *Acer nikoense*, which displayed tyrosinase inhibition and cytotoxicity to B16F1 cells. However, it had not been known that RD-containing cosmetics could induce leukoderma on the sites of application until the Kanebo's July 4, 2013 press release.

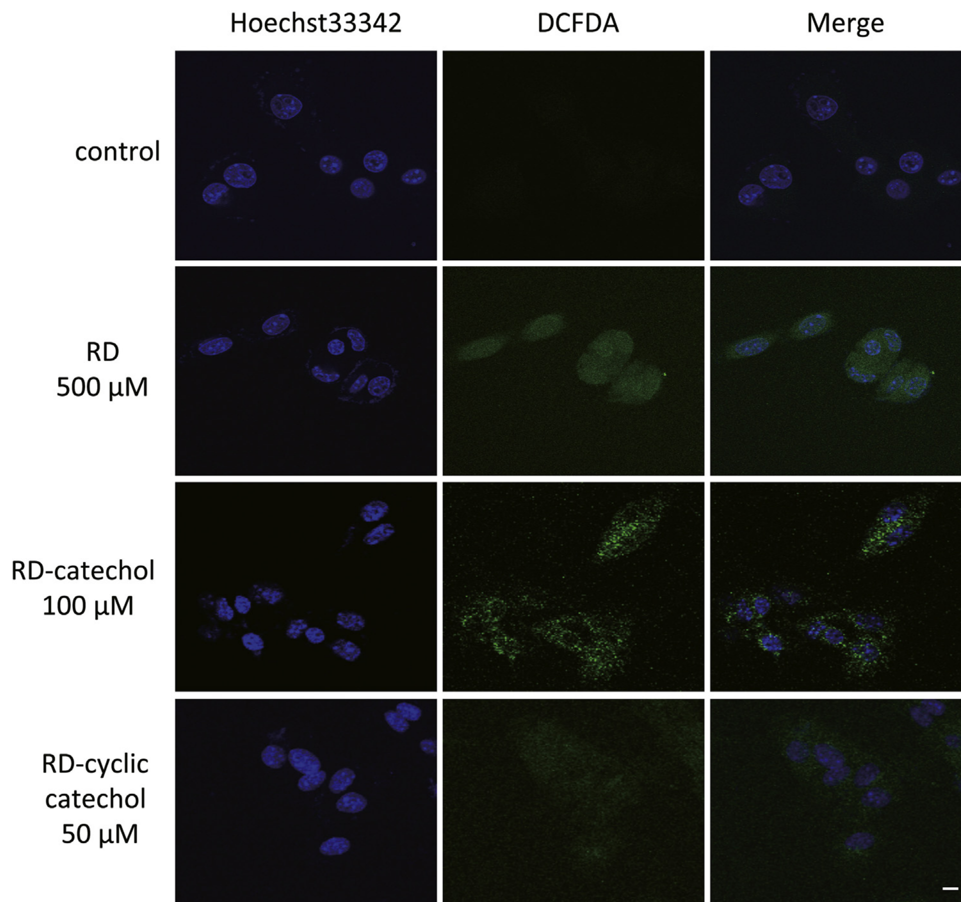

Fig. 6. The fluorescence microphotographs of ROS in B16F1 cells treated with RD and its metabolites. The cells were treated with RD, RD-catechol or RD-cyclic catechol and stained with CM-H2DCFDA and Hochst33342 that reacted with ROS (green, observed in the DCFDA+RD and DCFDA+RD-catechol cells) and the nuclei of the cells (blue), respectively. Scale bar 10 μm.

RD had a relatively strong inhibitory effect on the enzymatic activity of cellular tyrosinase prepared from melanocytes exposed to RD [17,18]. According to our mushroom tyrosinase assay, the inhibitory activity of tyrosinase by RD (IC50: 89.7 μM) was comparable to hydroquinone (IC50: 94.6 μM) but weaker than resveratrol (IC50: 23.0 μM) (data not shown). The growth inhibition assay showed that the proliferation of pigmented human melanocytes (NHEMb) and B16F1 cells was clearly suppressed by RD, while that of low-pigmented human melanocytes (NHEMa) was not. This was consistent with other previous results [17,18]. Among the phenolic compounds tested, the cytotoxicity of RD to NHEMb and B16F1 cells was milder than hydroquinone and resveratrol. Consequently, it is difficult to distinguish leukoderma-inducible compounds from non-inducible ones by the combination of tyrosinase assay and growth inhibition assay.

To elucidate further the molecular mechanism of cytotoxicity by RD, we have analyzed RD-treated cells by flow cytometry. Although both the NHEMb and B16F1 cells generated ROS when they were exposed to RD for 48 h, their subG1 fractions were not clearly increased and activated caspase 3 could not be detected. Interestingly, the frequency of S and G2/M phase cells increased significantly in the RD-treated B16F1 but not significantly in the NHEMb cells. The difference in the S and G2/M phases between the two cells is not clear, but it might be related to the duration of the cell cycle between two cells. These results suggest that cytotoxicity of RD to melanocytic cells results from cell cycle arrest that can induce degradation of cells without apoptosis. Yang et al. [25] reported that in melanocytic cells treated with 300–900 μM RD, autophagosome makers LC3II and LAMP1 were markedly upregulated. The phenolic substances are good substrates for tyrosinase and produce reactive *ortho*-quinones that can bind to cellular thiol compounds such as cysteine and GSH through the sulfhydryl group [26–28]. We have also reported that RD-quinone and RD-cyclic quinone reacted rapidly with cellular thiols [19]. Moreover, we examined the metabolism of RD in B16F1 cells and confirmed the production of RD-pheomelanin and covalent binding of RD-quinone to cellular proteins [21]. Thus, it can be surmised that the interaction of RD-oxidative compounds with the cellular sulfhydryl group resulted in the cell cycle arrest and autophagy.

We have previously reported that a phenolic compound, *N*-propionyl-4-*S*-cysteaminylphenol (NPr-4-*S*-CAP) was selectively incorporated into the melanin biosynthesis cascade of melanocytic cells and induced apoptotic cell death through ROS production [22,29]. Recently, we have shown that RD is also incorporated into the tyrosinase-initiated biochemical pathway to produce oxidative metabolites [19,20]. It can therefore be estimated that tyrosinase-catalyzed products, RD-catechol and RD-cyclic catechol, exert cytotoxicity more strongly than RD. In the present study, we have shown that RD-catechol and RD-cyclic catechol possessed cytotoxicity in B16F1, NHEMa and NHEMb cells more strongly than RD. In particular, growth of NHEMa and NHEMb was completely suppressed by a concentration of RD metabolites as low as 100 μM (Supplementary Fig. 2), indicating RD-catechol and RD-cyclic catechol is >10 fold more cytotoxic to NHEM cells.

Supplementary Fig. 2 related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.07.010.

Unlike RD-catechol, RD-cyclic catechol did not produce ROS but showed increased amounts of the subG1 fraction. Further analyses are required to elucidate why these two metabolites showed different biochemical activities. The results obtained suggest that RD-oxidized metabolites and ROS production are at least partially responsible for cell degradation. Catechols are known to be cytotoxic through the production of ROS and/or the *ortho*-quinones, depending on their structure [30]. In the present study it was not examined whether the RD-derived *ortho*-quninones, RD-quinone and RD-cyclic quinone, are involved in the cytotoxicity of RD. Our recent study using B16F1 cells exposed to RD, RD-quinone thio-ether adducts were detected, while RD-catechol and RD-cyclic catechol were not detected at appreciable levels [21]. This may suggest that these catechols are rapidly oxidized in melanocytes, producing ROS [19]. The recent studies suggest that RD-mediated degradation of epidermal melanocytes induces host immunity involving CCR4+CCR8+ T cells [31,32]. It remains unclear whether a part of RD and/or RD metabolites or a related peptide could be presented on the MHC class 1 molecule to mediate host cell immunity.

Both NPr-4-*S*-CAP and RD have been shown to be oxidized by tyrosinase [19,20,28,33] and exert cytotoxicity to melanocytic cells. We suggest that, in addition to the assay for tyrosinase inhibition and B16F1 cell cytotoxicity, the phenolic or catechol compounds should be tested for their potential to be incorporated into the melanin biosynthetic pathway as a substrate of tyrosinase.

Conflict of interest

All the authors state no conflict of interest.

Acknowledgements

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