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Research Article

Terrein: a new melanogenesis inhibitor and its mechanism

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Abstract. Terrein is a bioactive fungal metabolite whose effects are almost unknown. In this study, we found for the first time that terrein has a strong hypopigmentary effect in a spontaneously immortalized mouse melanocyte cell line, Mel-Ab. Treatment of Mel-Ab cells with terrein (10–100 μM) for 4 days significantly reduced melanin levels in a dose-dependent manner. In addition, terrein at the same concentration also reduced tyrosinase activity. We then investigated whether terrein influences the extracellular signal-regulated protein kinase (ERK) pathway and the expression of microphthalmia-associated transcription factor (MITF), which is required for tyrosinase expression. Terrein was found to induce sustained ERK activation and MITF down-regulation, and luciferase assays showed that terrein inhibits MITF promoter activity in a dose-dependent manner. To elucidate the correlation between ERK pathway activation and a decreased MITF transcriptional level, PD98059, a specific inhibitor of the ERK pathway, was applied before terrein treatment and found to abrogate the terrein-induced MITF attenuation. Terrein also reduced the tyrosinase protein level for at least 72 h. These results suggest that terrein reduces melanin synthesis by reducing tyrosinase production via ERK activation, and that this is followed by MITF down-regulation.

Key words. Terrein; Melanogenesis; Tyrosinase; MITF; ERK.

In mammals, melanin production is restricted to the melanocytes of the skin, hair follicles, and pigment epithelium in the retina. Three melanocyte-specific enzymes, tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2 are involved in melanogenesis, in which tyrosine is converted into melanin pigments. In particular, tyrosinase catalyzes two rate-limiting steps in melanin synthesis: the hydroxylation of tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and the oxidation of DOPA to dopaquinone [1].

Fungi have provided us with many important biomedical substances. For example, kojic acid, used as a cosmetic agent for skin whitening, was isolated from *Aspergillus oryzae* [2]. However, safer and more effective skin-whitening agents are needed because of the carcinogenic potential and the weak whitening effect of kojic acid [3, 4]. To identity a new class of cosmetic agents, we previously screened for melanin biosynthesis inhibitors among fungal metabolites and reported on the tyrosinase inhibitors, melanocins A–C, produced by *Eupenicillium shearii* [5]. During the course of our continuous screening for these melanin biosynthesis inhibitors, we isolated terrein from *Penicillium* species. Terrein has a relatively

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simple structure and can be easily synthesized [6]. However, the biologic effects of terrein are comparatively unknown.

Microphthalmia-associated transcription factor (MITF) is a basic helix-loop-helix-leucine-zipper (bHLHZip) transcription factor, which regulates melanocyte pigmentation, proliferation, and survival [7]. At least five MITF isoforms are present in humans. Among these, M-MITF is involved in the transactivation of melanocyte-specific genes encoding melanogenic enzymes, such as tyrosinase, TRP-1, and TRP-2 by binding with the M-box in the promoter regions of these genes [8, 9].

Extracellular signal-regulated kinase (ERK) plays a pivotal role in cell proliferation and differentiation [10, 11], and ERK pathway activation has been reported to be related to a reduction in melanin synthesis [12]. Moreover, MITF phosphorylation via ERK activation is linked to the degradation of MITF [13, 14]. In a recent study, we also demonstrated that sphingosine-1-phosphate inhibits melanin synthesis by activating ERK in Mel-Ab cells [15]. Therefore, we investigated the effects of terrein on melanogenesis in Mel-Ab cells with respect to ERK pathway activation.

Materials and methods

Materials

Fatty acid-free bovine serum albumin (BSA), 12-O-tetradecanoylphorbol-13-acetate (TPA), cholera toxin (CT), synthetic melanin, L-DOPA, α-melanocyte-stimulating hormone (α-MSH), and mushroom tyrosinase were purchased from Sigma (St. Louis, Mo.).

Extraction of terrein

Terrein was extracted from the culture broth of a microorganism identified as *Penicillium* species 20135. Briefly, the culture broth was extracted with 80% acetone and the extract was concentrated in vacuo, and then with an equal volume of EtOAc three times; the EtOAc extract was then concentrated in vacuo to dryness. The crude extract obtained was subjected to SiO_2 (Merck, Darmstadt, Germany) column chromatography followed by stepwise elution using $CHCl_3$-MeOH (50:1, 20:1, 10:1). The active fractions eluted by $CHCl_3$-MeOH (20:1) were pooled and concentrated in vacuo. The residue obtained was applied to a Sephadex LH-20 column and then eluted with MeOH to give the purified compound, at a purity exceeding 99% by high-performance liquid chromatography using an ODS column (YMC C_{18}) eluted with MeOH:H_2O (30:70). Based on electron spray ionization-mass spectrometry, ^{1}H-NMR, and ^{13}C-NMR, heteronuclear multiple quantum coherence, and heteronuclear multiple-bond correlation spectral data, the purified compound was verified as terrein ($C_8H_{10}O_3$; mol, wt 154.06 kDa) [16].

Antibodies

Antibodies recognizing phospho-specific ERK1/2 (Thr-202/Tyr204, No. 9101S), total (phosphorylated and non-phosphorylated) ERK1/2 (No. 9102), phospho-specific Akt (Ser473, No. 9271), and phospho-specific CREB (Ser133, No. 9196) were purchased from Cell Signaling Technology (Beverly, Mass.); microphthalmia Ab-1 (C5, MS-771-P0) from NeoMarkers (Fremont, Calif.); and tyrosinase (C-19) and actin (I-19) antibodies from Santa Cruz Biotechnology (Santa Cruz, Calif.).

Cell cultures

Mel-Ab is a mouse-derived spontaneously immortalized melanocyte cell line that produces large amounts of melanin [17]. Mel-Ab cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS), 100 nM TPA, 1 nM CT, 50 µg/ml streptomycin, and 50 U/ml penicillin at 37 °C in 5% CO_2. B16/F10 murine melanoma cells were incubated in DMEM supplemented with 10% FBS, 50 µg/ml streptomycin, and 50 U/ml penicillin at 37 °C in a 5% CO_2 atmosphere.

Cell viability assay

Cell viability was determined using a crystal violet assay [17]. After incubating cells with terrein for 24 h, the culture medium was removed, and the cells were stained with 0.1% crystal violet in 10% ethanol for 5 min at room temperature, and then rinsed four times. The crystal violet retained by adherent cells was then extracted with 95% ethanol. Absorbance was determined at 590 nm using an ELISA reader (TECAN, Salzburg, Austria).

Measurement of melanin content, and microscopy

Melanin contents were measured as described previously [18] with slight modification. Briefly, cells were treated with terrein at various concentrations for 4 days. Cell pellets were dissolved in 1 ml of 1 N NaOH at 100 °C for 30 min and then centrifuged for 20 min at 16,000 × *g*. The optical densities (OD) of the supernatants were measured at 400 nm using an ELISA reader. Standard curves of synthetic melanin (0–300 µg/ml) were prepared in triplicate for each experiment. Before measuring the melanin content, cells were observed under a phase contrast microscope (Olympus, Tokyo, Japan) and photographed using a CoolSNAP$_{cf}$ digital video camera system (Roper Scientific, Tucson, Ariz.) supported by RS Image software (Roper Scientific).

Tyrosinase activity

Tyrosinase activity was determined as previously described [19] with slight modification. Briefly, Mel-Ab cells were cultured in six-well plates. After incubating with terrein at various concentrations (10–100 µM) for 4 days, the cells were washed with ice-cold PBS and lysed with phosphate buffer (pH 6.8) containing 1% Triton

X-100. The cells were then disrupted by freezing and thawing, and lysates were clarified by centrifugation at 10,000 × *g* for 5 min. After quantifying protein levels and adjusting concentrations with lysis buffer, 90 µl of each lysate, containing the same amount of protein, was placed in each well of a 96-well plate, and 10 µl of 10 mM L-DOPA was then added per well. Control wells contained 90 µl lysis buffer and 10 µl of 10 mM L-DOPA. After incubation at 37 °C, absorbance was measured every 10 min for at least 1 h at 475 nm using an ELISA reader.

A cell-free assay system was used to test for the direct effects of terrein on tyrosinase activity. Seventy microliters of phosphate buffer containing various concentrations of terrein were mixed either with 20 µl of 10 µg/ml mushroom tyrosinase or with 20 µl of human tyrosinase containing 20 mg of total protein extracted from primary cultured human melanocytes; 10 µl of 10 mM L-DOPA was then added to each well. Following incubation at 37 °C, absorbance was measured at 475 nm.

Western blot analysis

Cells were lysed in cell lysis buffer [62.5 mM Tris-HCl, pH 6.8, 2% SDS, 5% β-mercaptoethanol, 2 mM phenylmethylsulfonyl fluoride, protease inhibitors (Complete, Roche, Mannheim, Germany), 1 mM Na_3VO_4, 50 mM NaF, and 10 mM EDTA]. Ten micrograms of protein per lane was then separated by SDS-polyacrylamide gel electrophoresis and blotted onto PVDF membranes, which were saturated with 5% dried milk in Tris-buffered saline containing 0.4% Tween 20. Blots were incubated with the appropriate primary antibodies at a dilution of 1:1000, and then further incubated with horseradish peroxidase-conjugated secondary antibody. Bound antibodies were detected using an enhanced chemiluminescence plus kit (Amersham International, Little Chalfont, UK). Results were analyzed using a Bio-Rad (Hercules, Calif.) GS-700 imaging densitometer.

Transfection and luciferase assay

B16 melanoma cells were cultured in six-well plates, and transfected using the GenePORTER transfection reagent, according to the manufacturer's recommendations (Gene Therapy Systems, San Diego, Calif.). Luciferase reporter plasmid pMITF was kindly provided by Dr. R. Ballotti (Nice, France) [20]. To determine the effect of terrein, 2 µg/well of the reporter plasmid was transfected with 1 µg/well of pSV β-galactosidase vector (Promega, Madison, Wis.), to control for transfection efficiency variations. Forty-eight hours after transfection, the cells were washed with PBS and lysed with lysis buffer using a Luciferase Assay Kit (Applied Biosystems, Bedford, Mass.), according to the manufacturer's recommendations. Soluble extracts were harvested and assayed for luciferase and β-galactosidase activities.

Statistics

Differences between results were assessed for significance using Student's *t* test.

Results

Terrein has no cytotoxic effect in Mel-Ab cells

The structure of terrein is shown in figure 1. While screening for substances that inhibit melanin biosynthesis in Mel-Ab cells, we detected strong activity among the fermentation metabolites of *Penicillium* species 20135. Activity-guided fractionation then led to the isolation of terrein, 4,5-dihydroxy-3-propenyl-2-cyclopenten-1-one. Although terrein was first isolated in 1935, its biological activities have rarely been studied, other than its antimicrobial effects [21]. To determine whether terrein has a cytotoxic effect on Mel-Ab cells, we applied terrein at 10–100 µM for 24 h, and measured cell viability using a crystal violet assay. As shown in figure 2, terrein was found to have no cytotoxic effect on Mel-Ab cells at the concentrations used.

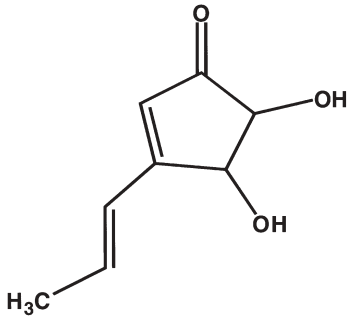

Figure 1. The structure of terrein.

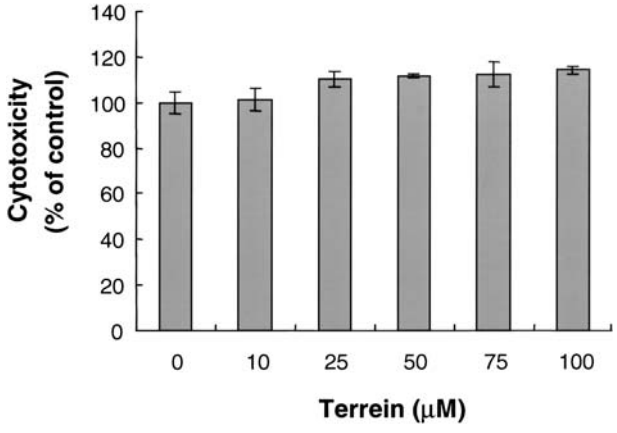

Figure 2. Effects of terrein on Mel-Ab cell viability. Cells were serum-starved for 24 h and terrein was added to serum-free medium at 10–100 µM for 24 h. Cell viabilities were determined by crystal violet assay. Each determination was made in triplicate and data shown are means ± SD.

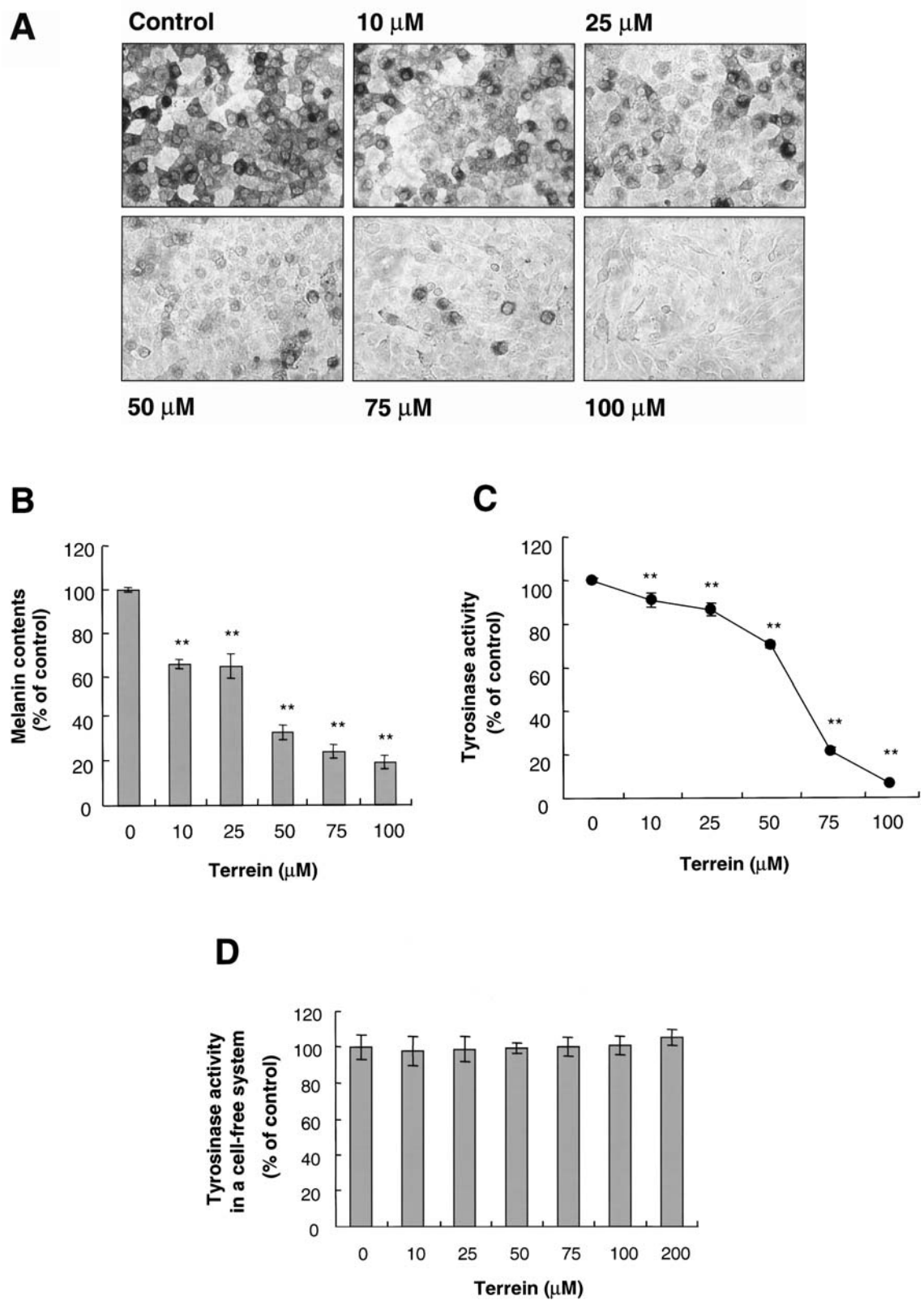

Figure 3. Effects of terrein on melanogenesis in Mel-Ab cells. Mel-Ab cells were cultured for 4 days in medium containing 10–100 μM terrein. Pictures were taken under a phase contrast microscope using a digital video camera (*A*). Melanin contents (*B*), tyrosinase activity (*C*) in Mel-Ab cells, and tyrosinase activity in a cell-free system (*D*) were measured, as described in Materials and methods. The results are averages of triplicate experiments and the data shown represent means ± SD. ** $p < 0.01$ compared to the untreated control.

Terrein has a strong hypopigmentary effect on Mel-Ab cells

Terrein was tested at 10–100 μM for 4 days, and Mel-Ab cells were then photographed under a phase contrast microscope. Terrein-treated cells were much less pigmented than untreated cells (fig. 3A). We also examined the melanin contents of Mel-Ab cells after 4 days of terrein treatment at 10–100 μM. In agreement with the microscopic observations, melanin levels were significantly reduced in a dose-dependent manner by terrein treatment (fig. 3B). In addition, terrein at the same concentrations also decreased tyrosinase activity in Mel-Ab cells (fig. 3C). These findings suggest that terrein negatively regulates tyrosinase and subsequently suppresses melanin synthesis in Mel-Ab cells.

Many skin-whitening agents inhibit tyrosinase directly. Thus, to investigate the direct effects of terrein on tyrosinase, we measured the tyrosinase activities of human and mushroom tyrosinase in a cell-free system, as described in Materials and methods. Terrein was found to have no direct inhibitory effect on human tyrosinase activity at concentrations ranging from 10 to 200 μM (fig. 3D), and an identical result was obtained using mushroom tyrosinase (data not shown).

Terrein induces the persistent activation of ERK and reduces MITF protein levels

To elucidate the mechanism underlying the hypopigmentary effect of terrein, changes in melanogenesis-related signals induced by terrein were analyzed by Western blotting. Because the phosphorylation of ERK has been reported to trigger MITF degradation [22], we examined whether terrein induces ERK activation. As shown in figure 4, terrein induced sustained ERK activation for at least 6 h. In addition, we investigated whether ERK activation by terrein is correlated with MITF down-regulation. Our results also show that MITF protein was clearly attenuated from 30 min after terrein addition.

In addition, we examined cyclic AMP response element-binding protein (CREB), which is known to activate the MITF promoter. However, terrein had no effects on its phosphorylation level. Several studies have found that the Akt pathway is involved in the melanogenesis of G361 and B16 melanoma cells [19, 20]. However, we found no evidence for Akt pathway activation by terrein in these cells, i.e., no changes in phospho-Akt levels were observed.

Terrein suppresses the transcriptional activity of MITF

Changes in MITF transcriptional activity were investigated by luciferase assay. To examine MITF promoter activity, the luciferase reporter plasmid pMITF was transfected into B16 melanoma cells, terrein was then applied for 48 h, and luciferase activity was measured. As shown in figure 5, MITF transcriptional activity was reduced to 60±12.9% of the control level after adding 10 μM terrein, and to 39±4.6% with 100 μM. These results indicate that terrein reduces MITF expression by inhibiting MITF transcriptional activity.

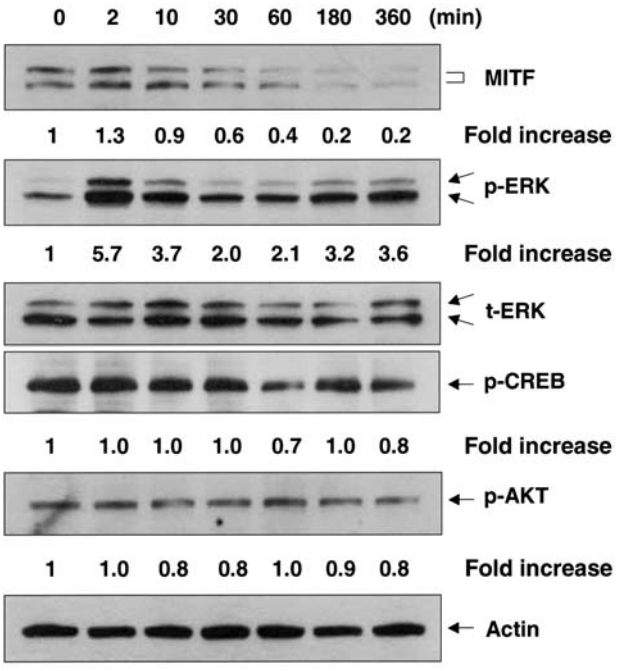

Figure 4. Terrein induces ERK activation, and down-regulates MITF. After 24 h of serum starvation, Mel-Ab cells were treated with terrein at the times indicated. Whole-cell lysates were then subjected to Western blot analysis using antibodies against MITF, phospho-specific ERK (p-ERK), phospho-specific CREB (p-CREB), and phospho-specific Akt (p-AKT). Equal protein loadings were confirmed using phosphorylation-independent ERK (t-ERK) or anti-actin antibody. Fold increases over the control were determined by densitometric analysis, and results are shown below each lane.

Terrein-induced MITF down-regulation is correlated with ERK activation

Terrein down-regulated MITF by reducing transcriptional activity and also activated the ERK pathway. To investigate the relationship between the level of MITF and the ERK pathway, Mel-Ab cells were treated with the ERK pathway inhibitor PD98059 for 1 h before terrein treatment. This treatment abrogated terrein-induced MITF down-regulation (fig. 6).

Terrein reduced tyrosinase protein levels in Mel-Ab cells

MITF has been reported to bind to the M-box within the tyrosinase promoter and thus up-regulate tyrosinase gene expression [9]. In the present study, we found that terrein does not directly reduce tyrosinase activity in a

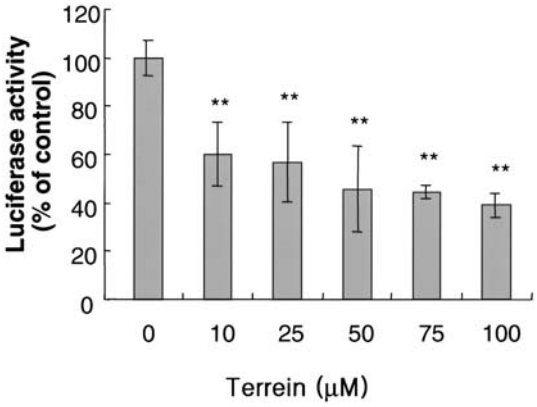

Figure 5. Terrein down-regulates MITF promoter activity. B16 cells were transfected with 2 μg of luciferase reporter plasmid and 1 μg pSV-β-galactosidase control vector. Cells were then incubated with terrein at 10–100 μM. Luciferase activities were measured as recommended by the manufacturer, and data were normalized with respect to β-galactosidase activity. Results are expressed as percentages of the untreated control. Each determination was made in triplicate and the data shown represent means ±SD. ** $p<0.01$ compared to the untreated control.

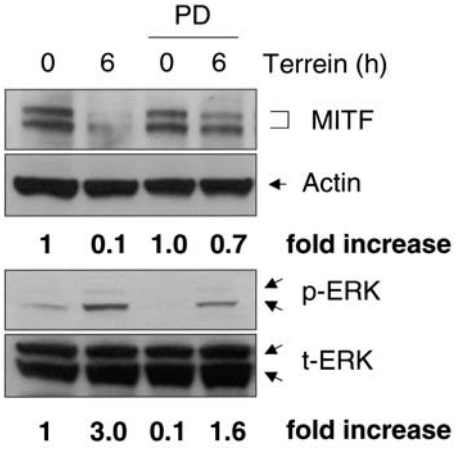

Figure 6. MITF down-regulation by terrein is correlated with ERK activation. Mel-Ab cells were starved in serum-free medium for 24 h, and then either pretreated or not with PD98059 for 1 h before terrein was applied for 6 h at 100 μM. Western blotting for MITF and phospho-specific ERK (p-ERK) was then performed with whole-cell lysates. Equal protein loadings were confirmed using phosphorylation-independent ERK (t-ERK) or anti-actin antibody. Fold increases over the control were determined by densitometric analysis, and results are shown below each lane.

cell-free system, but does reduce tyrosinase activity in Mel-Ab cells. Therefore, we investigated whether terrein affects tyrosinase expression in a time-dependent manner by Western blot analysis. As shown in figure 7, tyrosinase levels were clearly reduced 24–72 h after terrein treatment. These results suggest that terrein inhibits tyrosinase not by inhibiting its activity but by reducing the amount of tyrosinase at the protein level.

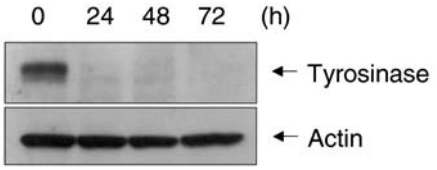

Figure 7. Terrein decreases the protein level of tyrosinase in Mel-Ab cells. Cells were cultured with 100 μM of terrein for 24–72 h, and whole-cell lysates were then subjected to Western blot analysis with antibody against tyrosinase. Equal protein loadings were confirmed using anti-actin antibody.

Discussion

Terrein was first isolated as a metabolite of *A. terreus* in 1935 [23] and was reported to be produced by other species of *Aspergillus* and by *Penicillium raistrickii* [24]. In the present study, terrein was extracted from *Penicillium* species 20135. Terrein has weak antibacterial activity against *Escherichia coli, Entercoccus faecalis, Bacillus subtilis*, and *Staphylococcus aureus* [21]. Moreover, terrein is an intermediate for the preparation of C series prostaglandins and analogs of other prostaglandins [25]. However, relatively little is known about terrein, and its effects are almost unknown. In the present study, we investigated whether terrein has a hypopigmentary effect in Mel-Ab cells, and found that terrein strongly down-regulates melanin synthesis by reducing tyrosinase production, and not by directly inhibiting tyrosinase activity. MITF plays a pivotal role in melanocyte pigmentation, proliferation, and survival [7]. In humans, mutations in the MITF gene cause Waardenburg syndrome type IIA, which is characterized by abnormal skin and hair pigmentation [26, 27]. Moreover, MITF is known to be a transcription factor of the melanogenic enzymes tyrosinase, TRP-1, and TRP-2 [9, 28].

The ERK pathway is a membrane-to-nucleus signaling cascade involved in cell proliferation, cell differentiation, and apoptosis [29, 30], and it plays a critical role in melanogenesis. Previous studies have shown that ERK activation is related to cAMP-induced melanogenesis in B16 melanoma cells [31]. However, other studies found that constitutive mutants of Ras and MEK inhibit tyrosinase transcription [12]. We also found that PD98059 increases melanogenesis in human melanocytes [32] and in Mel-Ab cells [15]. In addition, activated ERK is known to phosphorylate MITF at serine 73, and RSK-1 (activated by ERK) phosphorylates MITF at serine 409. Phosphorylated MITF has also been reported as a target for proteolysis through the ubiquitin-dependent proteasome pathway [13, 22].

The present study shows that terrein activates ERK and reduces MITF protein levels. To our knowledge, this is the first demonstration of the activation of ERK by ter-

rein. The terrein receptor has not yet been reported. Moreover, because of its hydrophilic structure, terrein is unlikely to penetrate the cell membrane. Thus, to elucidate the mechanism of ERK activation by terrein, further work is required on the terrein receptor. To investigate the relationship between ERK activation and MITF down-regulation, we pretreated with PD98059 before terrein treatment and re-examined MITF levels, and found that ERK activation is involved in the down-regulation of MITF. Although phosphorylated ERK peaked at 2 min, and was sustained for at least 6 h, MITF degradation occurred after 30 min of terrein treatment. The discrepancy between the kinetics of ERK phosphorylation and MITF degradation is thought to be due to the speed of ERK phosphorylation compared to that of proteasomal degradation, which requires some time because it occurs via ubiquitination which follows ERK activation.

Changes in MITF transcriptional activity were investigated by luciferase assay. After 48 h terrein treatment, the transcriptional activity of MITF was significantly reduced. We therefore checked the level of CREB, a MITF transcription factor. CREB is a critical regulator of immediate early gene transcription, and is phosphorylated in response to several growth factors and stress signals. Moreover, mitogen-activated protein kinases (MAPKs) are involved in the phosphorylation of CREB [33]. However, phosphorylated CREB levels were found to be similar. Terrein may, therefore, inhibit other MITF transcription factors.

On the other hand, a specific inhibitor of the Akt pathway, LY294002, stimulates melanin synthesis in the B16 melanoma cell line [20]. The Akt signaling pathway has been reported to be involved in the melanogenesis of G361 melanoma cells [34]. However, we found no difference in phospho-Akt levels after terrein treatment, which suggests that the Akt pathway does not participate in the terrein-induced inhibition of melanogenesis.

In summary, we investigated the hypopigmentary effect of terrein and its underlying mechanism. Terrein is a simple compound based on the cyclopenten skeleton. In this study, we found that terrein has a considerable ability to inhibit melanin biosynthesis but has no cytotoxic activity. Our results suggest that terrein inhibits melanogenesis in Mel-Ab cells by down-regulating MITF via ERK activation leading to the inhibition of tyrosinase production. Thus, terrein has potential as a skin-whitening agent. To further study its structure-activity relationships, we are synthesizing terrein and a range of terrein derivatives, and intend to examine the skin-whitening effect of terrein in an animal model.

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