マウス Ni アレルギーモデルの惹起相における自然免疫の役割

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A. 研究目的

本研究班の遠藤康男らによって開発されたマウス Ni アレルギーモデル(Sato *et al.*; *Clin Exp Allergy* 37:743-751, 2007)は金属アレルギー成立機序の解明に資する動物モデルとして注目されている。アレルギー反応は感作相 priming (or sensitization) phase と惹起相 elicitation phase からなっている。遠藤らは LPS が感作相で Ni アレルギーの成立を著しく促進するばかりでなく、惹起相の炎症反応も促進・増強することを報告している。本研究では、惹起相に着目して種々の菌体成分、特に歯周病関連細菌に特徴的な Toll-like receptor (TLR)2 リガンドの効果を検討した。

B. 材料と方法

$NiCl_2$ 溶液 (1 mM) と LPS (*E. coli*) 溶液の 1:1 混合液を BALB/c マウス (6-7 週齢雌) に腹腔内注射し (0.25 ml/ mouse)、10 日後に $NiCl_2$ 溶液とテスト標品の 1:1 混合液を耳介に皮内注射 (20 μl) して、以後その腫脹を測定した。なおテスト標品として、*E. coli* LPS の他、*P. intermedia* ATCC 25611 LPS 画分、*P. gingivalis* 381 の LPS 画分とリポペプチド画分、合成 MDP (NOD2 リガンド)ならびに FK565 (NOD1 リガンド)を供試した。また、TLR2 KO マウスを供試する実験も実施した。

C. 結果

①*E. coli* LPS の濃度 (0.04 –1 μg/ml) 依存的に耳介反応が増強された。②$NiCl_2$ 溶液による惹起注射に先立って *E. coli* LPS を投与すると同時投与に比較して更に反応は増強された。特に 4 時間前投与が最強の作用を示した。③*P. intermedia* LPS 画分、*P. gingivalis* LPS 画分とリポペプチド画分、MDP および FK565 (各 100 μg/ml)も明確な増強作用を示した。④*P. intermedia* ならびに *P. gingivalis* 由来標品の作用は TLR2 KO マウスでは殆ど認められなくなったが、*E. coli* LPS は TLR2 KO マウスでも通常のマウスと同程度の作用を示した。

D. 考察

①様々な菌体成分が惹起反応を増強することが証明された。実験成績から、TLR4 リガンド(*E. coli* LPS) に加えて、少なくとも TLR2 リガンド(後述)、NOD1 ならびに NOD2 リガンドにも増強作用が認められた。②TLR2 KO マウスを供試した実験結果は、歯周病原細菌と目される *P. intermedia* ならびに *P. gingivalis* の LPS 画分が *P. gingivalis* のリポペプチド画分と同様に TLR2 依存的に作用していることを示している。この成績は、両菌 LPS 画分の作用が LPS そのものよりもむしろ混入したリポペプチドに起因することを示唆している。③歯周病が動脈硬化を始めとして様々な全身疾患を増悪するとの知見が集積している。その機序に歯周病関連菌の TLR2 活性化作用が係わるとの説が有力視されている。今回の成績は、歯周病関連菌の TLR2 活性化因子としてのリポペプチドの重要性を裏付ける成績といえる。

E. 結論

自然免疫を活性化する各種 TLR 系ならびに NOD 系リガンドが、マウス Ni アレルギーモデルの惹起反応を増強した。特に歯周病関連細菌の *P. intermedia* ならびに *P. gingivalis* の菌体成分では TLR2 リガンドのリポペプチドの重要性が示された。

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骨髄由来樹状細胞の移入による金属アレルギーの感作成立とTLRシグナリング

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A．研究目的

T細胞性金属アレルギーの発症では、金属イオンの情報を持つMHC classⅡ-ペプチド複合体を発現する抗原提示細胞によるT細胞への抗原提示が重要なステップとして位置付けられているが、金属アレルギーを解析するモデル動物が存在しなかったことから、いまだ不明な点が多い。最も強力な抗原提示能力を持つ樹状細胞（以下、DC）は、Toll様受容体（TLR）ファミリー分子を発現しているため、各種病原体成分により活性化されると、T細胞への抗原提示に関与するCD80、CD86、MHC class Ⅱなどの発現レベルが増大することから、金属アレルギーの発症にもDCが深く関与していることが推測される。我々の研究班では、金属アレルギー発症マウスモデルの作製に成功している。そこで、本研究では、このマウスモデル実験系を用いて、金属アレルギー発症におけるDCの関与と、TLR signalingの役割を明らかにすることを目的とする実験を行った。

B．方法

C57BL/6マウスの骨髄細胞をGM-CSF（10 ng/ml）で10日間処理し、骨髄由来DCを得た。この細胞を$PdCl_2$(0.2 mM)、または$PdCl_2$+LPS（10 ng/ml）で24時間処理した。細胞を洗浄して$PdCl_2$及びLPSを除去した後、B6マウス（7週齢雌）に$5x10^5$ cells/マウスの量で移入した。10日後にPBSまたは$PdCl_2$溶液（1 mM）をマウスの右耳の耳介に皮内注（15 μl）して、以後その腫脹を24時間おきに四日間測定した。細胞表面のCD11c、CD80、CD86、MHC class Ⅱ分子の発現レベルはflow cytometryを用いて解析した。

C．結果

PBSでchallengeしたマウスにおいて、未処理DC移入群、$PdCl_2$処理DC移入群、及び$PdCl_2$ + LPS処理DC移入群の間に優位な腫脹の差は観察されなかった。一方、$PdCl_2$でchallengeしたマウスにおいて、$PdCl_2$+LPS処理DC移入群は、未処理DC移入群や$PdCl_2$処理DC移入群と比較して優位な腫脹の増大を示した。$PdCl_2$処理DC移入群は、未処理DC移入群と比較して48時間以降の腫脹が増大する傾向を示した。なお、皮内注を行わなかった左耳に関しては、全てのマウスでいかなる腫脹も観察されなかった。

D．考察

$PdCl_2$ + LPS処理DC移入群が未処理DC移入群よりも優位な腫脹の増大を示したことから、in vitroで金属イオンを処理したDCを移入することにより感作を成立させることが可能であることが示唆された。また、$PdCl_2$単独では感作成立が不十分であったことから、感作の成立にはTLR signalingの活性化、すなわち自然免疫反応の活性化が重要であることが示唆された。骨髄由来DCにおいて、LPS-TLR4 signalingの活性化により、CD80、CD86、MHC class Ⅱ分子の発現が効果的に増強することと合わせて、in vitroで金属イオンの情報を持つMHC class Ⅱ-ペプチド複合体を細胞表面に発現する活性型DCが得られることが示唆された。このin vitro金属イオン-LPS処理DCが金属イオンと反応するT細胞の同定や検出に利用可能かどうかを調べるために、金属アレルギーをすでに発症したマウスのT細胞との反応性を検討することが今後の課題である。

E．結論

In vitroで金属イオンとTLRリガンドを同時処理した骨髄由来DCを移入することにより、金属アレルギーの感作が成立することがわかった。

IV. 研究成果の刊行に関する一覧表

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V. 研究成果の刊行物・別刷

DOI:10.1111/j.1600-0625.2007.00618.x
www.blackwellpublishing.com/EXD

Original Article

Antimelanogenesis effect of Tunisian herb *Thymelaea hirsuta* extract on B16 murine melanoma cells

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Accepted for publication 11 July 2007

Abstract: Skin pigmentation is the result of melanogenesis that occurs in melanocytes and/or melanoma cells. Although melanogenesis is necessary for the prevention of DNA damage and cancer caused by UV irradiation, excessive accumulation of melanin can also cause melanoma. Thus, we focused on the antimelanogenesis effect of an extract from *Thymelaea hirsuta*, a Tunisian herb. Murine melanoma B16 cells were treated with *T. hirsuta* extract, and then cell viability and synthesized melanin content were measured. We found that the *T. hirsuta* extract decreased the synthesized melanin content in B16 cells without cytotoxicity. Tyrosinase is a key enzyme of melanogenesis and extracellular signal-regulated kinase (ERK)-1/2 phosphorylation is known to be related to melanogenesis inhibition. To clarify its mechanism, we also determined ERK1/2 phosphorylation and tyrosinase expression level. ERK1/2 was immediately phosphorylated in cells just after treatment with the extract. The tyrosinase expression was inhibited after 24 h of stimulation with the extract. The *T. hirsuta* extract was fractionated, and we found that one fraction considerably decreased the melanin synthesis in B16 cells and that this fraction contains daphnanes as the main component. This indicates that our findings might be attributable to daphnanes.

Key words: antimelanogenesis – B16 melanoma – tyrosinase – ERK1/2 – *Thymelaea hirsuta*

Please cite this paper as: Antimelanogenesis effect of Tunisian herb *Thymelaea hirsuta* extract on B16 murine melanoma cells. Experimental Dermatology 2007; 16: 977–984.

Introduction

In mammals, melanin is synthesized in the melanosomes of melanocytes. Its synthesis is regulated by melanogenic enzymes such as tyrosine, tyrosinase-related protein 1 (TRP-1) and tyrosinase-related protein 2 (TRP-2) (1–3). Tyrosinase is a copper enzyme and a key enzyme in the process of melanogenesis from L-tyrosine to 3,4-dihydroxyphenylalanine (DOPA), and then it catalyses the oxidation of DOPA into DOPA quinine. TRP-2, which functions as DOPA-chrome tautomerase, catalyses the rearrangement of DOPA-chrome into 5,6-dihydroxyindole-2-carboxylic acid (DHICA), and TRP-1 oxidizes DHICA into a carboxylated indole-quinone. The stimulation of tyrosinase activity is thus related to melanogenesis.

Thymelaea is a genus comprising about 30 species of evergreen shrubs under the flowering plant family *Thymelaeaceae*, which is native to the Canary Island, the Mediterranean region, north of central Europe, and east of central Asia. In this report, we focused on *T. hirsuta*, which is native to North Africa. In the Republic of Tunisia, this plant has been used traditionally as an antiseptic and for the treatment of hypertension. However, its traditional medicinal uses are not based on scientific research or investigation, and other effects have not been clarified.

The Republic of Tunisia is located in North Africa, and its north side faces the Mediterranean Sea while its south side leads to the Sahara. Between the Mediterranean Sea and the Sahara, the distance is only 100–350 km. This means that the dryness gradient is very high and plants in this area might have some antistress factors in their system, because plants that have evolved in this area could not have survived without accumulating these factors. Thus, we focused on this area and screened Tunisian medicinal plants for unique and new biological activities. We decided to evaluate the biological activity of *T. hirsuta* extract in relation to the maintenance of skin homeostasis, particularly for melanogenesis as a new activity.

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Recently, the antimelanogenesis and/or melanogenesis stimulation effect by plant extracts have been reported (4–13). Many of these articles focused only on tyrosinase activity and expression levels. In this study, we focused on not only tyrosinase expression, but also on the intracellular signal transduction cascade. The antimelanogenesis effect of the *T. hirsuta* extract on cultured murine B16 melanoma cells was investigated.

Materials and methods

Extraction of *T. hirsuta*

Aerial leaves of *T. hirsuta* (10 g) were extracted with 70% EtOH (100 ml) for 1–2 weeks at room temperature. After extraction, the extract was filter-sterilized using a 0.45 μm pore size filter (Millipore, Billerica, MA, USA) and then stored at −80°C until use.

Cells and cell culture

Mouse B16 melanoma cells (4A-5) were purchased from the Riken Cell Bank (Tsukuba, Japan). B16 mouse melanoma cells were maintained as a monolayer culture in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 10% fetal bovine serum (Sigma, St Louis, MO, USA), 4 mM L-glutamine (Sigma), 50 units/ml penicillin and 50 μg/ml streptomycin (Cambrex, East Rutherford, NJ, USA) at 37°C in a humidified atmosphere of 5% CO_2.

Measurement of melanin content

The melanin content was measured by a modification of the method of Hosoi et al. (14). B16 melanoma cells were seeded onto 100-mm dishes at a density of 5×10^5 cells per dish and cultivated by the method described above. After overnight incubation, the medium was replaced with sample-containing medium (200-, 2000- and 20 000-fold dilution or 0.5 μg/ml of sample) and incubated further for 2 days. The medium was then removed, and then the cells were washed twice with phosphate-buffered saline (PBS) and harvested by trypsinization (0.25% trypsin/0.02% EDTA in PBS; Sigma). The harvested cells were pelleted and the cell membrane was dissolved using 0.1% Triton X-100. The synthesized melanin was then purified and precipitated in 10% trichloroacetate. The purified melanin was dissolved by incubation in 8 N NaOH for 2 h at 80°C. The absorbance of the solution was measured at 410 nm and the melanin content was calculated using a standard curve for synthetic melanin.

Western blotting

B16 melanoma cells were seeded onto 100-mm dishes at a density of 3×10^6 cells per dish and cultivated by the method described above. After overnight incubation, the medium was replaced with 300-fold-diluted sample-containing medium followed by incubation for 0.5, 1, 2, 5, 24 or 48 h. The medium was then removed, the cells were washed twice with PBS and total protein was extracted using RIPA buffer (Sigma) according to the manufacturer's instructions. Fifteen micrograms of extracted protein sample was resolved by 12% (for activated ERK1/2 and ERK2) or 10% (for tyrosinase) sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose membrane, and blotted with anti-activated ERK1/2 monoclonal antibody (Sigma; 1:1785 dilution), anti-ERK2 monoclonal antibody (Sigma; 1:660 dilution), and anti-tyrosinase polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:200 dilution). The signal was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore), after reaction with labelled anti-mouse IgG_1 antibody (for activated ERK1/2 and ERK1/2; Zymed Laboratories Inc., San Francisco, CA, USA; 1:1000 dilution) or HRP-labelled anti-rabbit IgG antibody (for tyrosinase; Santa Cruz Biotechnology, Inc.; 1:2000 dilution). As a loading control for the assay on tyrosinase expression, the SDS-PAGE gels were stained with Coomassie brilliant blue (CBB), as follows. After SDS-PAGE, the gels were fixed twice for 15 min each time with a fixation solution containing 40% (v/v) acetic acid and 50% (v/v) MeOH. The fixed gels were then stained with CBB (Wako, Osaka, Japan).

Cell viability assay

Cell viability was determined with Guava PCA (GE Healthcare, UK Ltd, Buckinghamshire, UK) using the ViaCount program for analysis. B16 murine melanoma cells were seeded onto 100 mm dishes at a density of 5.0×10^5 cells per dish and cultivated by the method described above. After overnight incubation, the medium was replaced with sample-containing medium (200-, 2000- and 20 000-fold dilution) and further incubated for 2 days. After medium removal, the cells were washed twice with PBS and harvested by trypsinization. The harvested cells were resuspended in growth medium and stained with the ViaCount reagent (GE Healthcare, UK Ltd) according to the manufacturer's instructions. The ViaCount reagent differentially stains viable and non-viable cells based on their permeability to the DNA-binding dyes in the reagent. The fluorescence of each dye is resolved operationally, allowing the quantitative assessment of viable and non-viable nucleated cells present in a suspension. The system counts the stained nucleated events, and then uses the forward scatter properties to distinguish free nuclei and cellular debris from cells.

F-actin staining

F-actin polymerization was detected by fluorescent dye staining of B16 cells using phalloidin-rhodamine. B16 murine melanoma cells were subcultured onto four-well chamber slides (Nunc, Rochester, NY, USA) at a density of 4.0×10^4

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cells per well. After overnight incubation, the medium was replaced with sample-containing medium (1000-fold dilution) and further incubated for 24 h. After medium removal, the cells were washed with PBS and fixed with 3.7% formaldehyde for 5 min. After fixation, the cells were washed and permeabilized with 0.2% Triton X-100 in PBS for 5 min, and then washed three times. Rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO, USA) was added to the cells at 70 nM for 30 min at room temperature in the dark and the cells were then washed thrice with PBS. Mounting reagent containing DAPI (Vector Laboratories, Inc., Burlingame, CA, USA) was then added and fluorescent signals were then detected and photographed using a Leica DMI-4000B fluorescence microscope and a Leica DFC300FX CCD camera (Leica, Wetzlar, Germany). The images were analysed using the Leica Application Suite (Leica).

Fractionation of *T. hirsuta* extract

Aerial leaves (100 g) of *T. hirsuta* were extracted with MeOH (1.3 l) and evaporated to dryness *in vacuo* at 30°C. The MeOH extract (8.03 g) was then partitioned between EtOAc (500 ml × 3) and H_2O (500 ml). The H_2O-soluble portion was partitioned with BuOH (500 ml × 3). The EtOAc-soluble portion (1.30 g) was fractionated into 17 fractions (*Th*-EtOAc-1–17) using a silica gel column ($\varphi\xi$ 2.2 × 33 cm, hexane/EtOAc, 5:1 → 2:1 → $CHCl_3$/MeOH, 8:1 → 0:100). *Th*-EtOAc-11 was separated into six fractions (*Th*-EtOAc-11-1–6) by a C_{18} Sep-Pak cartridge (MeOH/H_2O, 50:50 → 100:0 → $CHCl_3$/MeOH, 1:6 → 100:0 water).

Extraction and isolation of daphnanes from *T. hirsuta*

Leaves (100 g) of *T. hirsuta* were extracted with MeOH (1.3 l) and evaporated to dryness *in vacuo* at 30°C. The MeOH extract (8.0 g) was then partitioned between EtOAc (500 ml × 3) and H_2O (500 ml). The H_2O-soluble portion was partitioned with BuOH (500 ml × 3). The EtOAc-soluble material (1.30 g) was subjected to a silica gel column (φ 2.2 × 33 cm, hexane/EtOAc, 5:1 → 2:1 → $CHCl_3$/MeOH, 8:1 → 0:100) to afford 17 fractions (*Th*-EtOAc-1–17). *Th*-EtOAc-11 was separated into six fractions (*Th*-EtOAc-11-1–6) by a C_{18} Sep-Pak cartridge (MeOH/H_2O, 50:50 → 100:0 → $CHCl_3$/MeOH, 1:6 → 100:0; Waters, Milford, MA, USA). *Th*-EtOAc-11-3 was purified by reversed-phase C_{18} HPLC [Inertsil ODS3 (1.0 × 25 cm), flow rate 2.0 ml/min; solvent CH_3CN/H_2O (65:35)] to give two daphnane diterpenoids, *Th*-EtOAc-11-3-6 and *Th*-EtOAc-11-3-8.

Structural analysis

^{1}H-NMR spectra were measured and recorded using a Brucker AVANCE 500 spectrometer and EIMS was recorded on an MS-6890.

Th-EtOAc-11-3-6 (genkwadaphnin): EIMS *m/z* 602 (M^+), ^{1}H-NMR ($CDCl_3$, 500 MHz): δ7.60–7.75 (m, aromatic-H), 7.50 (s, 1H), 5.28 (s, 1H), 5.05 (s, 2H), 4.95 (d, J = 2.5 Hz, 1H), 4.25 (s, 1H), 3.90 (m, 1H), 3.85 (d, J = 5.4 Hz, 2H), 3.63 (br s, 1H), 2.63 (q, J = 7.3 Hz, 1H), 1.88 (s, 3H), 1.75 (d, J = 2.6 Hz, 3H), and 0.88 (m, 3H).

Th-EtOAc-11-3-8 (gnidicin): EIMS *m/z* 628 (M^+), ^{1}H-NMR ($CDCl_3$, 500 MHz): δ7.60–7.75 (m, aromatic-H), 7.50 (s, 1-H), 6.37 (d, J = 16.0 Hz, 1H), 5.18 (s, 1H), 5.05 (s, 2H), 4.95 (d, J = 2.5 Hz, 1H), 4.25 (s, 1H), 3.90 (m, 1H), 3.85 (d, J = 5.4 Hz, 2H), 3.63 (br s, 1H), 2.52 (q, J = 7.3 Hz, 1H), 1.88 (s, 3H), 1.75 (d, J = 2.6 Hz, 3H), and 0.88(m, 3H).

Statistics

Differences between means were assessed for significance using the Student's *t*-test.

Results

Inhibitory effect of *T. hirsuta* extract on melanin synthesis in B16 melanoma cells without cytotoxicity

The effect of *T. hirsuta* extract on the melanogenesis of B16 mouse melanoma cells was examined. We observed that B16 mouse melanoma cells treated with *T. hirsuta* extract showed a time-dependent decrease in cytoplasmic accumulation of melanin (Fig. 1a). We quantified the synthesized melanin content per cell and found that the extract inhibited melanin synthesis by more than 50% versus control (Fig. 1b) in a dose-dependent manner, suggesting that the extract can inhibit melanin synthesis in B16 mouse melanoma cells.

To determine the cytotoxicity of *T. hirsuta* extract on B16 murine melanoma cells, we performed the cell viability assay using the Guava ViaCount system. From this assay, we found that the extract is not cytotoxic to B16 murine melanoma cells after 48 h of incubation (Fig. 1b). Observation of the cells treated with the extract using a phase-contrast microscope also showed the absence of cytotoxicity (Fig. 1a).

These results suggest that the extract has antimelanogenesis effect on B16 cells without cytotoxicity. Moreover, cells incubated with the extract for more than 24 h showed the ability to undergo mitosis without melanin accumulation.

Melanogenesis inhibition by *T. hirsuta* extract via MAPK activation

Recently, ERK1/2, a key molecule in the mitogen-activated protein kinase (MAPK) intracellular signal transduction cascade, has been reported to be associated with melanogenesis. The phosphorylation of ERK1/2 contributes to antimelanogenesis. To determine the mechanism of

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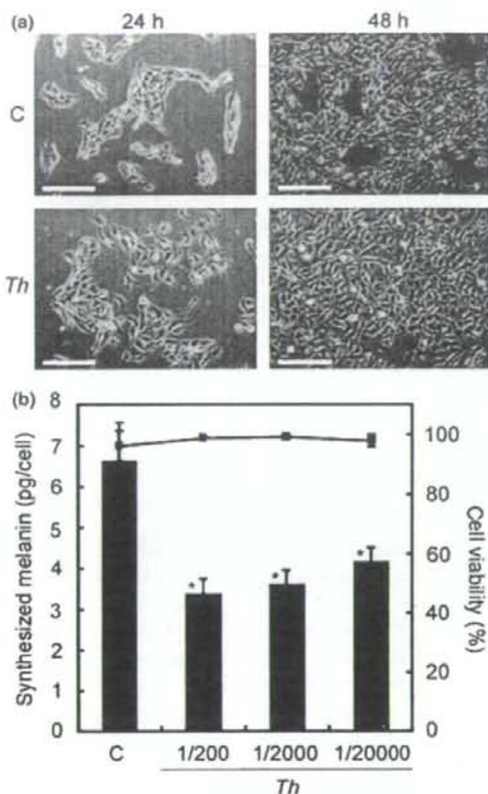

Figure 1. Effect of *Thymelaea hirsuta* extract on melanogenesis in B16 murine melanoma cells. (a) Change in shape of B16 cells treated with *T. hirsuta* extract. B16 cells were seeded at a density of 5×10^5 cells per 100-mm dish. After overnight incubation, the cells were treated with (*Th*, lower panels) or without (C, upper panels) *T. hirsuta* extract at 2000-fold dilution for 24 h (left panels) or 48 h (right panels). All photographs were taken at 100× magnification and each bar represents 200 μm. (b) Synthesized melanin content and cell viability of B16 murine melanoma cells treated with *T. hirsuta* extract. B16 cells were seeded at a density of 5×10^5 cells per 100-mm dish. After overnight incubation, the cells were treated with (*Th*) or without (control) *T. hirsuta* extract at the indicated dilution for 48 h. The synthesized melanin was then extracted and measured as described in the Materials and methods. The quantified melanin content was divided by the viable cell number. The treated cells were also assayed for cell viability as described in the Materials and methods. The bar graph indicates melanin content (left-hand *y*-axis). The line graph indicates cell viability (right-hand *y*-axis). Results represent means ± SD of triplicate samples. *Statistically significant ($P < 0.001$) difference between *T. hirsuta* extract-treated cells and control.

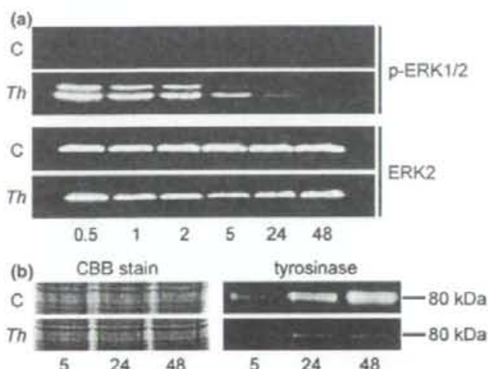

Figure 2. Effect of *T. hirsuta* extract on protein expression by B16 murine melanoma cells. (a) ERK1/2 phosphorylation in B16 murine melanoma cells treated with *T. hirsuta* extract. B16 cells were seeded at a density of 3×10^6 cells per 100-mm dish. After overnight incubation, the cells were treated with (*Th*) or without (C) *T. hirsuta* extract at 300-fold dilution for indicated time. Total proteins were then extracted and resolved by SDS-PAGE and the resolved proteins were then blotted onto a nitrocellulose membrane. The phosphorylated ERK1/2 extracted from the B16 cells was detected by immunoblotting with anti-activated ERK1/2 monoclonal antibody (upper panel) and anti-ERK1/2 monoclonal antibody (lower panel). The signal was visualized using the Immobilon Western Chemiluminescent HRP Substrate. The numbers below the panels indicate treatment time (h). (b) Tyrosinase expression levels in B16 murine melanoma cells. B16 cells were seeded at a density of 3×10^6 cells per 100-mm dish. After overnight incubation, the cells were treated with (*Th*) or without (C) *T. hirsuta* extract at 300-fold dilution for 5, 24 and 48 h. Total proteins were then extracted and resolved by SDS-PAGE; the resolved proteins were then blotted onto a nitrocellulose membrane (right panel) or stained with CBB dye as loading control (left panel). The tyrosinase extracted from B16 cells was detected by immunoblotting with anti-tyrosinase polyclonal antibody. The signal was visualized using the Immobilon Western Chemiluminescent HRP Substrate.

antimelanogenesis in B16 cells treated with the extract, we performed Western blotting to determine ERK1/2 phosphorylation (Fig. 2a).

Total protein was extracted from B16 murine melanoma cells treated with or without the *T. hirsuta* extract. To obtain sufficient protein for Western blotting, more cells are required. Thus, we used 3×10^6 cells as the initial cell number. To maintain the same treatment condition as that of the melanin quantification assay, the B16 cells were treated with the *T. hirsuta* extract at 300-fold dilution.

Although the expression levels of ERK2 in cultured B16 cells were not affected by treatment with the extract (Fig. 2a, lower panel), the phosphorylation of ERK1/2 in B16 cells was considerably enhanced after 0.5–5 h of treatment with the extract (Fig. 2a, upper panel). This result indicated that the antimelanogenesis effect of the extract in B16 cells is associated with ERK1/2 phosphorylation. The MAPK cascade is one of the most important intracellular signal transduction cascades, and many important signals are transduced to the nucleus via this cascade. Based on the phosphorylation of ERK1/2 after a short treatment time, the antimelanogenesis component might be able to interact directly with ERK1/2. The results showing the

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relationship between the antimelanogenesis effect of *T. hirsuta* and ERK1/2 phosphorylation suggest that the extract may contain components that can stimulate ERK1/2 phosphorylation or regulate growth factor expression, transcription and translation.

Downregulation of tyrosinase expression by *T. hirsuta* extract

To determine the tyrosinase expression level of cells treated with *T. hirsuta* extract, we performed Western blot analysis (Fig. 2b). The tyrosinase expression levels of non-treated control cells as well as those treated for 5 h with 300-fold diluted extract were similar (Fig. 2b, right upper panel). However, cells treated for 24 and 48 h with the extract showed considerable reductions in tyrosinase expression levels (Fig. 2b, right lower panel).

These results suggest that the antimelanogenesis effect of the extract on B16 cells is associated with the downregulation of tyrosinase, the most important enzyme in melanogenesis. Moreover, the downregulation of tyrosinase by the extract requires a long treatment time of more than 5 h, and the downregulated tyrosinase expression level continues for at least 48 h.

Major compounds having antimelanogenesis activity in the *T. hirsuta* extract

To identify the major compound responsible for the activity, leaves of *T. hirsuta* was extracted in MeOH and the extract was partitioned with EtOAc, BuOH and water. We examined the effect of each layer on the melanogenesis of B16 cells and found that the EtOAc layer showed a dose-dependent antimelanogenesis activity (data not shown). The EtOAc layer was subjected to SiO_2 column chromatography to afford 17 fractions (*Th*-EtOAc-1–17) and *Th*-EtOAc-11 possessed the highest antimelanogenesis activity (data not shown). *Th*-EtOAc-11 was further fractionated into six fractions (*Th*-EtOAc-11-1–6) by a Sep-Pak ODS and *Th*-EtOAc-11-3 strongly inhibited melanin synthesis (Fig. 3). The NMR analysis revealed that the main component of the *Th*-EtOAc-11-3 fraction showed patterns which are identical to those of the typical charts of daphnane diterpenoids (15). The *Th*-EtOAc-11-3 was further separated by reversed-phase HPLC to give 15 fractions (*Th*-EtOAc-11-3-1–15) and each fraction was analysed by ^{1}H-NMR and EIMS. *Th*-EtOAc-11-3-6: EIMS m/z 602 (M^+), ^{1}H-NMR ($CDCl_3$, 500 MHz): δ7.60–7.75 (m, aromatic-H), 7.50 (s, 1H), 5.28 (s, 1H), 5.05 (s, 2H), 4.95 (d, J = 2.5 Hz, 1H), 4.25 (s, 1H), 3.90 (m, 1H), 3.85 (d, J = 5.4 Hz, 2H), 3.63 (br s, 1H), 2.63 (q, J = 7.3 Hz, 1H), 1.88 (s, 3H), 1.75 (d, J = 2.6 Hz, 3H), and 0.88 (m, 3H). Comparison of these data with previously published data indicated that *Th*-EtOAc-11-3-6 was identified with genkwadaphnin (16). *Th*-EtOAc-11-3-8: EIMS m/z 628 (M^+), ^{1}H-NMR ($CDCl_3$, 500 MHz): δ7.60–7.75 (m, aromatic-H), 7.50 (s, 1-H), 6.37 (d, J = 16.0 Hz, 1H), 5.18 (s, 1H), 5.05 (s, 2H), 4.95 (d, J = 2.5 Hz, 1H), 4.25 (s, 1H), 3.90 (m, 1H), 3.85 (d, J = 5.4 Hz, 2H), 3.63 (br s, 1H), 2.52 (q, J = 7.3 Hz, 1H), 1.88 (s, 3H), 1.75 (d, J = 2.6 Hz, 3H), and 0.88(m, 3H). Comparison of these data with previously published data revealed that *Th*-EtOAc-11-3-8 was identified with gnidicin (15).

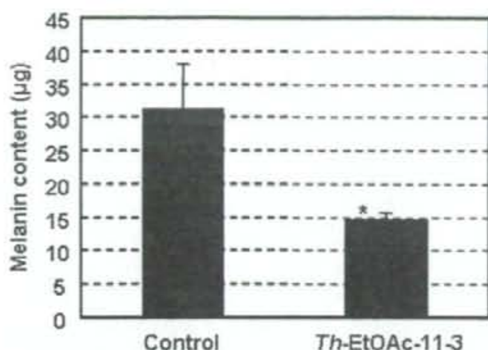

Figure 3. Synthesized melanin content in B16 murine melanoma cells. B16 cells were seeded at a density of 5×10^5 cells per 100-mm dish. After overnight incubation, the cells were treated with (*Th*-EtOAc-11-3) or without (control) fr.11-3 from the *T. hirsuta* EtOAc layer at a concentration of 0.5 µg/ml for 48 h. The synthesized melanin was then extracted and measured as described in the Materials and methods. Columns and bars represent means ± SD of triplicate samples. *Statistical significance ($P < 0.05$) between treated and control cells.

Discussion

Melanoma is the deadliest form of skin cancer and one of the most challenging of human cancers. This cancer is the fifth and sixth most common cancer among men and women, respectively. Recently, the demand for antimelanogenetic agents has increased all over the world, not only for anticancer uses but also for cosmetics as well. Choi et al. showed that an extract of *Lepidium apetalum* inhibited pigmentation via interleukin-6 signalling pathway (17). Solano et al. (2006) recently reviewed antimelanogenic agents and their specific molecular mechanisms, particurarly tyrosinase inhibition and antioxidative effects (18). Many researchers have also reported on the antimelanogenesis effects of some agents namely, 3,4-dihydroxyacetophenone (19), 4,4′-dihydroxybiphenyl (20), phospholipase D1 (21), phospholipase D2 (22), 4-*n*-butylresorcinol (23), miconazole (24), glycolic acid and lactic acid (25). This is because, depending on the age, melanin-rich spots occur on the skin and many people want to remove these spots. Thus, we would like to search for new agents that can regulate melanogenesis. There are many types of skin diseases aside from melanoma and melanin spots, such as vitiligo, porphyritic amiroidosis and hemochromatosis. Our main objective is

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to find new agents that can regulate melanogenesis from natural resources.

We investigated one plant having the ability to inhibit melanogenesis. An extract from the herb *T. hirsuta* considerably inhibited melanogenesis without cytotoxicity (Fig. 1). After 48 h of treatment with the extract, B16 cells were still completely viable (Fig. 1b), which is important if this plant will be used for medicine or cosmetics. If the *T. hirsuta* extract is cytotoxic even on murine cells, it would be difficult to market it as a product. Our findings showing an antimelanogenesis effect of the extract without cytotoxicity can lead to new treatment technologies.

In addition, the *T. hirsuta* extract downregulated the tyrosinase expression level after more than 24 h of treatment (Fig. 2b). The melanogenesis reaction is regulated by tyrosinase, a key enzyme and tyrosinase-related protein-1 and -2 (1–3). *Thymelaea hirsuta* extract downregulated the tyrosinase expression level and this effect continued for more than 48 h. This suggests that the actual compound that inhibits melanogenesis and downregulates tyrosinase expression might be the same and has a very strong influence on these activities. This is because the melanogenesis inhibition by the extract was observed after 24 h of treatment, and tyrosinase expression was also inhibited after 24 h. Furthermore, we found that tyrosinase expression almost completely disappeared even after 48 h. These results showing a long and continuous effect after only one treatment are useful for the management of disease or cosmetic problems, because the antimelanogenesis effect can be observed with a very low concentration of the extract applied only once.

Recently, the relationship between MAPK phosphorylation and melanogenesis inhibition has been shown (26–28). The antimelanogenesis effect of some agents has been shown to be associated with the phosphorylation of ERK1/2, one of the main molecules in the MAPK intracellular signal transduction cascade (29–31). Furthermore, transforming growth factor (TGF)-β1 has been shown to decrease melanin synthesis via ERK1/2 phosphorylation (32). Our results also showed ERK1/2 phosphorylation by the extract in B16 cells (Fig. 2a). This effect was observed after 0.5–5 h of treatment with the extract. The TGF-β1 effect was previously shown to be continuous for 6 h. The *T. hirsuta* extract target molecule and cascade might be the same as the TGF-β1 target, because our result is almost similar to the report in which ERK1/2 phosphorylation continued to occur after 6 h (31). On the other hand, it was clearly shown that corticotrophin-releasing hormone (CRH) phosphorylated ERK1/2 through CRH receptors in B16 cells within 15 min after treatment (33). Although it was shown that CRH treatment induces B16 cell migration via ERK1/2 phosphorylation (33) and it is quit different from our observation, it is possible that cell migration and antimelanogenesis share one signal transduction pathway. From these reports and our results, we propose three hypotheses for the ERK1/2 phosphorylation: (i) a compound in the *T. hirsuta* extract can phosphorylate ERK1/2 via the same cascade as that of TGF-β1, (ii) a compound in the *T. hirsuta* extract can phosphorylate ERK1/2 via the activation of CRH receptors; and (iii) a small compound in the *T. hirsuta* extract can phosphorylate ERK1/2 directly. As the extraction was carried out using organic solvent, however, the agent inducing this phosphorylation is predicted to be a small molecule. If indeed so, this actual component could pass through the cell membrane of B16 cells and phosphorylate the ERK1/2 directly. This is one reason behind the early phosphorylation of ERK1/2. These results suggest that the effect of tyrosinase on melanogenesis is downstream of the MAPK cascade.

To determine the main compound in the extract that is responsible for the antimelanogenesis effect, we carried out further fractionation and quantification of the melanin synthesized by B16 cells treated with each fraction. Results showed that one fraction of the EtOAc layer partitioned from the *T. hirsuta* extract, *Th*-EtOAc-11-3, has strong antimelanogenesis activity (Fig. 3). We further found that this fraction has two daphnanes, genkwadaphnin and gnidicin, as the main components. These results indicate that the antimelanogenesis effect of the extract on B16 cells might be attributable to these daphnanes. Daphnanes have been previously isolated from *Thymelaeaceae*, and it was previously reported that daphnanes can induce apoptosis in human leukaemia cells and also have neurotrophic and anticancer effects (34–36). However, the antimelanogenesis effect of daphnanes on B16 melanoma cells has never been reported. In addition, effects on intracellular signal transduction and tyrosinase expression of daphnanes have not been reported. From all of our results, we could hypothesize that daphnanes phosphorylate ERK1/2 at first, and tyrosinase expression is then downregulated by this signal via MAPK, followed by melanin synthesis inhibition. This is the first report showing that daphnanes might function as an antimelanogenesis agent through ERK1/2 phosphorylation and downregulation of tyrosinase in B16 murine melanoma cells.

We showed here for the first time that an herb grown in Tunisia has the ability to maintain skin homeostasis, particularly as an antimelanogenic agent. As another effect of the extract, we also observed that the extract induced cell shape change in B16 cells by changing the F-actin polymerization and distribution (Fig. 4). In the cells treated with the extract, phalloidin signals were strongly detected in the dendritic extensions (Fig. 4, lower-left panel), whereas phalloidin signals were detected widely in the cytoplasm of control cells (Fig. 4, upper-left panel). Moreover, while the control cells were clumped (Fig. 3, upper panels), the

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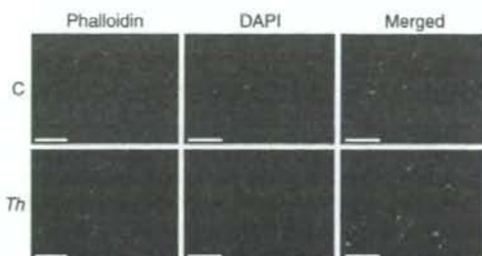

Figure 4. F-actin polymerization in B16 murine melanoma cells treated with *T. hirsuta* extract. B16 cells were seeded onto four-well chamber slides at 4×10^4 cells per well. After overnight incubation, the cells were treated with (*Th*) or without (C) *T. hirsuta* extract at 1000-fold dilution for 24 h. The cells were stained with rhodamine-phalloidin for the actin cytoskeleton (left panels) and with DAPI for the nuclei (middle panels). Merged images are shown in the right panels. All photographs were taken at 400× magnification and each bar represents 50 μm.

treated cells were separated (Fig. 3, lower panels). It is known that normal melanocytes in the basal layer of the epidermis have a dendritic cell shape and are scattered. We might be observing the effects of daphnanes on not only antimelanogenesis but also on some other important phenomena. To confirm the other effects of the extract, more analysis should be performed and more details of the mechanism should be clarified. We are now analysing the other effects of the *T. hirsuta* extract in detail.

From our results, we found that the *T. hirsuta* extract can inhibit melanin synthesis in B16 cells and this antimelanogenesis effect occurs via ERK1/2 phosphorylation and inhibition of tyrosinase expression. Furthermore, we suggested that these phenomena could be attributable to the daphnanes in the *T. hirsuta* extract. However, we do not discount the possibility that other minor compounds in the extract may also function as antimelanogenesis agents or that antimelanogenesis may be the result of a synergistic effect of some compounds in the extract. Nonetheless, our results suggest that compounds from the *T. hirsuta* extract can be useful antimelanogenesis agents.

Acknowledgements

This research was performed under the General Framework Agreement on Science and Technology Cooperation between the Ministry of Scientific Research, Technology and Competency Development (MRSTDC) of the Republic of Tunisia and the Alliance for Research on North Africa (ARENA) of the University of Tsukuba, Japan. The agreement, which was signed on the 6th of June 2005, is in line with the UN convention on Biological Diversity.

The authors express their gratitude to Dr Terence P.N. Talorete of the University of Tsukuba for his English correction.

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