Depigmentation of Melanocytes by the Treatment of Extracts from Traditional Chinese Herbs: A Cell Culture Assay

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Received January 23, 2006; accepted April 14, 2006

Objective: To obtain potential skin whitening agents from traditional Chinese herbs, we tested changes of melanin content in melanocyte cell lines after treatment with extracts of 90 traditional Chinese herbs. Methods: Mouse melanocyte cell lines were used. Depigmentation activity of the herb extracts was first screened in Mel-Ab cells, and then re-evaluated in melan-a cells and co-culture of melan-a and SP-1 cells. Melanin content and cell viability were the two indications for evaluation. Tyrosinase activity and the expression of melanin synthesis related enzymes in cells treated with the herb extracts were also tested. Results: Nine herb extracts were proved to have depigmentation activity similar to or better than that of arbutin and low cytotoxicity to melanocytes. Two of them were more effective in co-cultured melan-a cells. Most of the effective herb extracts inhibited tyrosinase activity and the expression of tyrosinase. Some of them also inhibited tyrosinase related protein-1 and/or tyrosinase related protein-2 in cultured cells. Conclusions: We have found 9 herb extracts to be promising skin whitening agents. Among them, water extract of Galla Chinensis and ethanol extract of Radix Clematidis exhibited higher depigmentation activity and caused lower tyrosinase activity in cell culture assays and are worthy to be further studied.

Key words traditional Chinese herb; depigmentation; melanogenesis; melanocyte; keratinocyte

Current therapies are still unsatisfied for skin pigmentation disease conditions. Furthermore, Asian women prefer lighter skin color and there is a great demand to develop more safe and effective skin whitening agents. Recently, many efforts have been paid to develop new therapeutic agents against pigmentation abnormalities, especially using novel biologically active compounds from natural plants. Many tyrosinase inhibitors that suppress melanogenesis have been actively studied with the aim of developing new whitening agents. Medicinal plants are most suitable for pharmacological research and drug development, because their constituents can be used not only as therapeutic agents but also as starting materials or models for the synthesis of drugs or pharmacologically active compounds. We are interested in re-evaluation of traditional Chinese herbs on melanogenesis from huge resources of famous whitening formulations and recipes in ancient literature. Traditional Chinese medicine has been used for the treatment of chloasma for a long history. Many famous ancient formulations and recipes are still in use to treat pigmentation disorders. Though these drugs were reported to be clinically effective in China, the mechanism and the active components have not been studied yet. Evaluation of Chinese herbal medicine in the treatment of skin pigmentation abnormalities may be beneficial for the development of new and more efficient remedies. In China, search for depigmentation medicine from traditional Chinese herbs is currently focused on those having inhibitory activity to tyrosinase. In a previous screening study performed by Japanese researchers with mushroom tyrosinase, several extracts of crude traditional Chinese drugs showed highly inhibitory activity. Results from these studies provided important information about traditional Chinese herbs on melanogenesis. However, melanin biosynthesis is a complicated process involving many factors including the key enzymes tyrosinase, tyrosinase related protein-1 (TRP-1), tyrosinase related protein-2 (TRP-2), cytokines from autocrine and paracrine and those related to melanin transportation and decomposition. Mushroom tyrosinase test is a simple method but with some disadvantages. Plant tyrosinase is different from mammalian tyrosinase because of its unique requirements for substrate and cofactor as well as its different sensitivity to inhibitors. Several papers published in Chinese indicated that many plant extracts showing inhibitory activity to mushroom tyrosinase in vitro did not reduce pigmentation activity in cells. Also, some compounds tested on mammalian tyrosinase did not give comparable results with mushroom tyrosinase. Thus, this study was undertaken to evaluate the depigmentation effect of traditional Chinese herbs based on melanocyte cell culture assays.

MATERIALS AND METHODS

Preparation of Herb Extracts Identification of the herbs used in this study was provided by the Union Bioengineering Institute (Beijing, China). The raw herbs were extracted with 95% ethanol and distilled water, respectively. Ethanol extracts were dissolved in PEH (50% propylene glycol, 30% ethanol and 20% distilled water) and water extracts were diluted in distilled water to 40 mg/ml as the stock solutions.

Cell Culture Mel-Ab Cell Culture: Mel-Ab cell is an immortalized and highly pigmented melanocyte cell derived from C57BL/6 mice (a kind gift from AmorePacific R&D center, Korea). The cells were cultured using the method described by Kim et al. Cells were placed into 24-well plastic culture plates (Corning, New York, NY, U.S.A.) at 1 × 10^5 cells/well. After incubated with 40 μg/ml tested herb extracts for 4 d, melanin content and cell viability of the con-
fluent Mel-Ab cells were tested using the protocol of Dooley et al.11) Melan-a Cell Culture: Murine melan-a melanocyte (melan-a cell) is originally derived from C57BL/6 J (black, a/a) mice, a kind gift from Prof. Dorothy C. Bennett (St George’s Hospital, London, U.K.). Melan-a cell is close to melanocytes in vivo in character and has been widely used as a suitable substitute for normal primary mouse melanocytes in melanin metabolism tests. Melan-a cells were maintained according to the method described by Bennett with minor modifications.12) The cells were seeded at 5 × 10^4 cells/well in six-well plates. Cells were treated with appropriate concentrations of herb extracts on day 2 and 5 and assayed on day 8. Arbutin, a known tyrosinase inhibitor from plant and a skin whitening agent, was used as a positive control.13) The assays were performed in triplicate twice. Treated melan-a cells were lysed in a extraction buffer containing 0.1 M Tris–HCl pH 7.2, 1% Nonidet-P40, 0.01% SDS and proteinase inhibitor cocktail (Sigma). After centrifugation, protein content was determined by the spectrophotometric measurement of formazan crystals in melan-a cells described above.14) The assays were evaluated by western blot assays at least in triplicate. The pellet was saved for melanin measurement.

Co-culture of Melan-a and SP-1 Cells: Murine SP-1 keratinocyte (SP-1 cell, a kind gift from prof. Vincent J. Hearing, NIH, U.S.A.) is derived from SENCAR mice.15) Co-culture system of melan-a and SP-1 cells was maintained using the method described by Yoon et al.15) Herb extract treatment and the assays after the treatment were the same as those for melan-a cells described above.

**Determination of Cell Viability** Crystal Violet Assay: The high melanin content within Mel-Ab cells may interfere with the spectrophotometric measurement of formazan crystals in MTT assay. Crystal violet staining was then used as an alternative for Mel-Ab cells to determine the percentage of viable cells as described before.10) The absorbance from crystal violet was measured at 590 nm using a microplate reader (Model 550, Bio-Rad).

MTT Assay: Melanin content in melan-a cells was much lower than that of Mel-Ab cells. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT, Sigma) assay was used to determine the viability of melan-a cells and the appropriate concentration of herb extract to treat cells.16) Herb extract of 100 μg/ml, 50 μg/ml, 25 μg/ml, 12.5 μg/ml, 6.25 μg/ml and 3.125 μg/ml diluted in culture medium were used to treat cells for 2 d. After incubated with MTT for 4 h, the optical density of each well was read at 540 nm using a microplate reader. The highest concentration with the cell survival rate over 75% or the next lower concentration of herb extract was chosen for further study.

**Measurement of Melanin Content in Cultured Cells** Melanin content was measured using the method of Ando with some modifications.17) Melanin amount in the well was obtained from the standard curve made by serial dilutions (0.05—0.25 mg/ml) of standard melanin (S. officinalis), and then divided by protein content of the well for calibration of cell number in the well. Depigmentation activity was represented by the percentage of melanin content in herb treated melan-a cells to that in untreated melan-a cells.

**Assay of Tyrosinase Activity in Melan-a Cells** Changes of tyrosinase activity in melan-a cells after treatment with 20 μg/ml herb extract were measured by L-dihydroxyphenylalanine (L-DOPA) staining of electrophoresed gel using the method described previously with some modifications.18) Lysate containing 11 μg protein in 22 μl mixed with equal volume of Tris–glycine native sample buffer (NOVEX, Invitrogen, U.S.A.) was loaded on a 10% polyacrylamide gel using Tris–glycine SDS running buffer (NOVEX, Invitrogen), and run at 120 V for 90 min. The gel was equilibrated in 200 ml rinse buffer (80 mM phosphate buffer, pH 6.8) for 30 min, and incubated in a staining solution (0.1% L-DOPA in 80 mM phosphate buffer) at 37°C in dark for 30 min. Tyrosinase activity was visualized in the gel as dark bands containing DOPA-melanin and measured by scanning the dark bands in a gel image analysis instrument (Bio-Rad gel doc 2000).

**Measurement of Tyrosinase, TRP-1 and TRP-2 in Melan-a Cells by Western Blot** Changes of melanin synthesis related proteins including tyrosinase, TRP-1 and TRP-2 in melan-a cells after treated with 20 μg/ml herb extract were evaluated by western blot. Cell extract containing 20 μg solubilized protein was separated in a 10% SDS-PAGE gel, and then transferred to a nitrocellulose membrane. After incubation with the blocking buffer, the membranes were incubated with primary polyclonal anti-tyrosinase antibody (SC-7833, Santa Cruz, U.S.A.), anti-TRP-1 antibody (SC-10443, Santa Cruz) and anti-TRP-2 antibody (SC-10452, Santa Cruz), and then incubated with the secondary antibodies horseradish peroxidase-conjugated rabbit anti-goat IgG antibody. Blotted antibody was visualized by chemiluminescence method (ECL kit, Amersham). Positive bands were analyzed using a gel image analysis instrument.

**Changes of Tyrosinase, TRP-1 and TRP-2 mRNAs Determined by Real-Time Quantitative RT-PCR** Melan-a cells were treated with 20 μg/ml herb extracts for 3 consecutive days. On day 4, total RNA from the cells was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, U.S.A.). First-strand cDNA was synthesized from total RNA by using oligo d(T)15 primer and AMV reverse transcriptase. Primers used for real-time quantitative PCR are: left primer 5'-gcaccacagaggtcg and right primer 5'-ttatggccgataggtgcatt for mouse tyrosinase, left primer 5'-aggcttcatactggtagctcaag and right primer 5'-ggctcatgtgtctgctctcag for mouse TRP-1, left primer 5'-tacatctggggactgga and right primer 5'-tggtcatcttgctgctg for mouse TRP-2, and left primer 5'-gagggtggtcacgactctatag and right primer 5'-tctccaaggaggagaggt for mouse β-actin. Amplicons were less than 150 bp in size. PCR mixture containing 10 pmol of each primer, 2.0 mM MgCl2, 100 mM/ml of each dNTP, 0.5 U of AmpliTaq Gold DNA polymerase, 2 μl of SYBR Green buffer (Applied Biosystems, Sparks, MD, U.S.A.) and 1.5 μl of cDNA sample in a total volume of 20 μl was run in a real-time quantitative PCR apparatus (Applied Biosystems 5700 Prism). Each sample was measured three times. Specificity of PCR product was examined by the dissociation curve of amplicons and the single band after agarose electrophoresis. Relative amount of the mRNA was calibrated by the amount of β-actin mRNA in the sample.

**Statistical Analysis** Values were expressed as mean±standard error (S.E.). Statistical significance of difference was examined by one-way ANOVA test.
RESULTS

Primary Screening of the 90 Herb Extracts in Mel-Ab Cells  Eighty-five herbs and five herb combinations frequently mentioned in the literature of Chinese traditional medicine for depigmentation treatment were selected for the screening. Depigmentation activity of the herb extracts is easily detected in the highly pigmented Mel-Ab cells. Therefore, we first used this cell for primary screening of the 90 herb extracts. According to the pioneering work of Curto et al., on the screening of safe and effective whitening products in Mel-Ab cell cultures, 40 μg/ml is sufficient for cell based primary screening. Herb extracts that satisfy the following criteria are worthy of further study: concentration of 50% inhibition of melanin synthesis (IC50)<100 μg/ml as well as low cytotoxicity with cell viability >75% at this concentration. Among the 90 herb extracts examined, only 16 extracts satisfied the two criteria (Table 1).

Evaluation of the 16 Herb Extracts from Primary Screening to Melan-a Cells  Melan-a cell is a non-tumorigenic mouse melanocyte containing less melanin than Mel-Ab, and having similar phenotype as the primary mouse melanocyte. The 16 herb extracts effective to Mel-Ab cells were therefore further examined using melan-a cells. Based on the cytotoxicity of these herb extracts, the concentrations selected for further evaluation were 40 μg/ml and 20 μg/ml or 20 μg/ml and 10 μg/ml. At the concentration of 20 μg/ml, 9 of the 16 herb extracts showed prominent depigmentation activity without remarkable cytotoxicity. Depigmentation activity at the concentration of 20 μg/ml was 44% for Rhizoma Chuan Xiong, 46% for Radix Cinnamomi, 47% for Ramulus Cinnamomi (at 10 μg/ml), 47% for Radix Ophiopogonis, 48% for Semen Gingko, 52% for Ligusticum sinense Oliv, 57% for Flos Carthami, 58% for Folium Perillae and 61% for Galla Chinensis compared to control (100%). That of the positive control arbutin was 63% (Fig. 1). For convenience, we will use numbers to represent the 9 unfamiliar herb names in the following text and figures (see Table 2).

Re-evaluation of the 9 Most Effective Herb Extracts to Co-culture of Melan-a and SP-1 Cells  Keratinocytes affect the proliferation and melanin metabolism of melanocytes in vivo. To exactly evaluate the depigmentation activity of the 9 herb extracts, we examined their activity once again using co-culture of melan-a and SP-1 cells. The concentration of herb extract used in the re-evaluation was

<table>
<thead>
<tr>
<th>Herb name</th>
<th>Concentration (μg/ml)</th>
<th>Depigmentation activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arbutin</td>
<td>10 20 100</td>
<td>63% (control)</td>
</tr>
<tr>
<td>Rhizoma Chuan Xiong</td>
<td>40 20 10</td>
<td>44%</td>
</tr>
<tr>
<td>Semen Gingko</td>
<td>40 20 10</td>
<td>48%</td>
</tr>
<tr>
<td>Radix Cinnamomi</td>
<td>40 20 10</td>
<td>47%</td>
</tr>
<tr>
<td>Ramulus Cinnamomi</td>
<td>40 20 10</td>
<td>47%</td>
</tr>
<tr>
<td>Galla Chinensis</td>
<td>40 20 10</td>
<td>61%</td>
</tr>
</tbody>
</table>

Table 2. Numbers Representing the Herb Extracts Mentioned in the Text and Figures

<table>
<thead>
<tr>
<th>Number</th>
<th>Latin name</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>Rhizoma Chuan Xiong</td>
</tr>
<tr>
<td>No. 2</td>
<td>Semen Gingko</td>
</tr>
<tr>
<td>No. 3</td>
<td>Radix Ophiopogonis</td>
</tr>
<tr>
<td>No. 4</td>
<td>Flos Carthami</td>
</tr>
<tr>
<td>No. 5</td>
<td>Folium Perillae</td>
</tr>
<tr>
<td>No. 6</td>
<td>Ramulus Cinnamomi</td>
</tr>
<tr>
<td>No. 7</td>
<td>Galla Chinensis</td>
</tr>
<tr>
<td>No. 8</td>
<td>Radix Cinnamomi</td>
</tr>
<tr>
<td>No. 9</td>
<td>Ligusticum sinense Oliv</td>
</tr>
</tbody>
</table>

Fig. 1. Depigmentation Activity of the 9 Herb Extracts to Melan-a Cells

From the results of MTT assay, herb extracts were tested at two concentrations of 40 μg/ml and 20 μg/ml or 20 μg/ml and 10 μg/ml in order that cytotoxicity was not present. No. 5 was cytotoxic at 20 μg/ml and only the result at 10 μg/ml was showed. Depigmentation activity was expressed as percentage of the control and each column represents mean±S.E. **p<0.01, as compared with that of the control. Refer to Table 2 for the represented herb extracts of No. 1—9 on abscissa. A: arbutin; C: negative control.
20 μg/ml and 10 μg/ml in order that cytotoxicity was not present. All the 9 herb extracts also showed some extent of depigmentation activity to co-culture of melan-a and SP-1 cells. Of them No. 8 and 9 herb extracts were more effective to co-culture system than to melan-a cells, with the depigmentation activity of 36% and 41% compared to control (100%), respectively. The positive control arbutin (20 μg/ml) had 73% of the control activity.

Effect of the 9 Herb Extracts on Tyrosinase Activity in Melan-a Cells

Change of tyrosinase activity in the cells treated with the herb extract is also a useful indication about its depigmentation activity to the cells. All of the 9 herbs inhibited the tyrosinase activity in melan-a cells to a certain degree (Fig. 3). Herb extracts of No. 9, 8, 5 and 3 were the most potent tyrosinase inhibitors which lowered the tyrosinase activity of melan-a cells to 20%, 31%, 52% and 61% of the control activity (100%), respectively. The positive control arbutin (20 μg/ml) caused 59% of the control activity.

Effect of the 9 Herb Extracts on Content of Tyrosinase, TRP-1 and TRP-2 Genes in Melan-a Cells

All the 9 herb extracts except No. 3 and 4 decreased the tyrosinase mRNA in melan-a cells to a significant degree (Fig. 5). All herb extracts except No. 1 and 9 lowered TRP-1 mRNA, and all herb extracts except No. 3 caused the decrease of TRP-2 mRNA in melan-a cells.

DISCUSSION

From recipes of traditional Chinese medicine for the treatment of hyperpigmentation diseases, we selected 90 herbs or herb formulations and prepared their ethanol and water extracts to evaluate their depigmentation activity. The 90 candidate herb extracts were first screened in Mel-Ab cells and then the effective herb extracts from the primary screening were assessed further in melan-a cells for their depigmentation activity and their cytotoxicity as well. Through the two screening procedures, we found 9 herb extracts, ethanol extracts of Rhizoma Chuan Xiong, Ligusticum sinense Oliv, Radix Ophiopogonis, Semen Gingko, Radix Clematidis, Folium Perillae and FIs Carthami at the concentration of 20 μg/ml, ethanol extracts of Ramulus Cinnamomi at the concentration of 10 μg/ml, and water extract of Galla Chinensis at the concentration of 20 μg/ml, to have significant depigmentation activity more than that of arbutin but without cytotoxicity in mouse melanocyte cell lines.
The 9 herb extracts showing higher depigmentation activity but without significant cytotoxicity were further tested in co-culture of melan-a and SP-1 cells. The epidermal melanin unit is composed of one melanocyte and approximate 36 neighboring keratinocytes working in concert to produce and distribute melanin. Keratinocytes produce paracrine factors that affect the proliferation, dendrite formation and melanin synthesis of melanocytes. Melanocytes cultured alone may not properly reflect melanin metabolism of the skin. We therefore used a melanocyte and keratinocyte co-culture system to mimic the in vivo condition. Among the 9 herb extracts, No. 8 and 9 herb extracts showed higher depigmentation activity to melan-a and SP-1 cells co-culture system than in melan-a cells. Therefore, the 2 herb extracts may also act on keratinocytes to provide indirect inhibitory effect on melanogenesis.

In this study, we also performed several preliminary experiments on the mechanisms of the 9 herb extracts. All the 9 herb extracts could decrease the melanin synthesis by inhibiting tyrosinase activity in cultured cells (Fig. 3). Decrease of tyrosinase activity, tyrosinase protein as well as its mRNA was found in melan-a cells treated with herb extracts of No. 5, 8 and 9, suggesting that they may down-regulate tyrosinase gene and thus lower tyrosinase activity and inhibit melanogenesis in the cells. Decrease of tyrosinase activity and tyrosinase mRNA but with normal content of tyrosinase was seen in cells treated with herb extracts No. 1, 2, 6 and 7, and decrease of tyrosinase activity and tyrosinase protein but with normal amount of its mRNA was found in cells treated with herb extract No. 3 and No. 4, indicating that these herb extracts may act on posttranslational modification and/or catabolism of tyrosinase protein in melan-a cells. For the positive control arbutin, decrease of tyrosinase activity without changes of tyrosinase protein and its mRNA found in melan-a cells is in accordance with the previous published data that it is simply a tyrosinase inhibitor.

However, there are several data without reasonable explanations at present time in results of this study. For example, No. 4 and 7 herb extracts showed higher depigmentation activity in lower concentration than in higher concentration (Fig. 2). Changes of TRP-1 and TRP-2 were not perfectly comparable to changes of their mRNA in No. 1, 3, 5, 6 and 9 herb extracts (Fig. 4, 5), suggesting that factors other than transcription rate of the genes lead to the changes of the proteins in cells treated with the herb extracts. Ingredients in an herb extract are very complicated, and different herb extract definitely has different mechanism for depigmentation activity. Only using purified effective components and performing more experiments can we know more about mechanisms for their depigmentation activities.

In summary, we screened 90 traditional Chinese herb extracts for their depigmentation activity. Nine of them showed definite characters of depigmentation activity, inhibition of tyrosinase activity, little or no cytotoxicity and lower effective concentrations in cell culture assays using mouse melanocyte Mel-Ab cell line, melan-a cell line as well as co-culture of melan-a and keratinocyte SP-1 cells. Further studies disclosed that water extract of Galla Chinensis (No. 8) and ethanol extract of Radix Clematidis (No. 9) exhibited higher depigmentation activity and caused lower tyrosinase activity in cell assays and are worthy to be investigated further using their purified components.

Acknowledgement This research project was sponsored by AmorePacific Corporation, Korea.

REFERENCES