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Use of non-melanocytic HEK293 cells stably expressing human tyrosinase for the screening of anti-melanogenic agents

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Synopsis

Tyrosinase (TYR) from mushrooms has been inappropriately used in the screening assay for hypopigmenting agents even though its biochemical properties are different from those of human TYR. Cell-free extracts of human epidermal melanocyes (HEMs) could be another choice for the assay, but HEMs grow too slowly to get a sufficient amount of cell-free extracts. In the present study, human embryonic kidney (HEK) 293 cells were transfected with a human TYR construct to establish a cell line that grows rapidly and expresses human TYR constitutively. Cell-free extracts of the established cell line, HEK293-TYR, were tentatively used in the screening assays for 11 phenylpropanoids that have chemical structures similar to that of L-tyrosine, the substrate of TYR. Of the 11 compounds, the strongest inhibition of TYR activity was shown by *p*-coumaric acid (IC_{50}, 3 μM), followed by 3-(4-hydroxyphenyl)propionic acid (50 μM) and 3-(4-hydroxyphenyl)lactic acid (70 μM). The results indicate that *p*-coumaric acid has an optimal chemical structure for the inhibition of TYR. The effects of these phenylpropanoids on melanin synthesis in HEMs correlated well with their effects on TYR activity *in vitro*. This study demonstrated that HEK293-TYR cells can be a good source of the human TYR enzymes needed in the screening assay of anti-melanogenic agents.

INTRODUCTION

Control of skin hyperpigmentation represents a major challenge in dermatology and cosmetics. Although both ablative and non-ablative laser-based techniques have been used to remove unwanted pigmentation including melasma, the results are not always consistent. In addition, no safe and effective pharmacological methods are currently available to combat hyperpigmentation.

The synthesis of melanin, a major pigment responsible for the coloration of the skin, hair, and eye, occurs principally in specialized organelles called melanosomes. Thus melanin

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synthesis is restricted to melanocytic cells that contain melanosomes. A number of enzymes including tyrosinase (TYR) are involved in the oxidative polymerization of the amino acid L-tyrosine to eumelanin or pheomelanin. TYR (EC 1.14.18.1) is a copper-containing enzyme that catalyzes two distinct reactions, i.e., the hydroxylation of L-tyrosine to dihydroxyphenylalanine (DOPA) and the subsequent two-electron oxidation to DOPA quinone (1,2). These reactions represent the rate-limiting steps of overall melanin synthesis, and therefore TYR inhibitors have been sought as potential hypopigmenting agents (3).

Mushroom TYR is often used as a substitute for human TYR in order to screen TYR inhibitors, probably because the latter is hardly available (4,5). However, the use of mushroom TYR for this purpose can be problematic because it is quite different from human TYR in terms of the amino acid sequence and substrate specificity (6–8). Indeed, many compounds have had inconsistent effects on the activity of TYR of mushroom, murine, or human origin (9–11). These observations have underscored the importance of using human TYR enzyme for the screening of hypopigmenting agents.

Under a situation where human TYR is not commercially available, cell-free extracts of human epidermal melanocytes (HEMs) have been used instead in the initial screening of TYR inhibitors (11). However, it has been difficult to obtain sufficient amounts of HEMs for this purpose because the cells grow very slowly. In addition, the culturing of HEMs requires a specialized medium that is very expensive. Highly proliferating melanin-producing melanoma cells may be a good alternative to HEMs, but they are not readily available either. Therefore, this study attempted to prepare a cell line that proliferates rapidly and expresses human TYR constitutively. Human embryonic kidney (HEK) 293 cells were chosen for this purpose because these cells are commercially available, relatively easy to culture *in vitro*, and widely used in the production of viruses or proteins. In the present study, HEK293 cells were stably transfected with a human TYR construct, and the cell-free extracts of the established cell line, HEK293-TYR, were tentatively used in the screening of potential TYR inhibitors.

MATERIALS AND METHODS

CHEMICALS

L-tyrosine and DOPA were purchased from Sigma-Aldrich (St. Louis, MO). The phenylpropanoids, including *p*-coumaric acid, *o*-coumaric acid, *m*-coumaric acid, cinnamic acid, *p*-methoxycinnamic acid, caffeic acid, ferulic acid, 3-(4-hydroxyphenyl)propionic acid, 3-(4-hydroxyphenyl)lactic acid, 3-(4-hydroxyphenyl)pyruvic acid, and 3-phenyllactic acid, were purchased from Sigma-Aldrich.

CELL CULTURE

Human embryonic kidney (HEK) 293 cells were purchased from American Type Culture Collection (Manassas, VA). The cells were cultured in the growth medium Dulbecco's minimum Eagle's medium (DMEM) containing 10% fetal bovine serum, 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B. The cells were cultured at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. HEMs,

derived from moderately pigmented neonatal foreskins, were obtained from Cascade Biologics (Portland, OR) and cultured with Medium 254 supplemented with human melanocyte growth supplement (Cascade Biologics), 100 U ml^{-1} penicillin, 0.1 mg ml^{-1} streptomycin, and 0.25 μg ml^{-1} amphotericin B.

DNA CONSTRUCTS

The pING-TYR plasmid that encodes human TYR under the control of the cytomegalovirus promoter (12,13) was kindly provided by Dr. Alan Houghton from Memorial Sloan-Kettering Cancer Center, and used as the template for the polymerase chain reaction (PCR)-amplification of the entire coding sequence of human TYR, using primers 5′-GGG **AAG CTT GCC ACC** ATG CTC CTG GCT GTT TTG TAC TGC C-3′ and 5′-GGG **TCT AGA** TTA TAA ATG GCT CTG ATA CAA GCT-3′ (*HindIII* and *XbaI* restriction sites were incorporated into the sense and antisense primers, respectively, as underlined). The sense primer also contained a Kozak sequence shown in bold font. PCR was done in a reaction mixture (50 μl) that contained 10 pmol of each primer, 1.25 units of Taq DNA polymerase, and 2.5 mM of dNTPs. The conditions for PCR were set as 1 min at 98°C, 35 cycles of 10 s at 98°C, 10 s at 65°C, and 3 min at 72°C, with a final extension step of 3 min at 72°C. The amplified product was purified by a PCR purification kit (SolGent, Daejeon, Korea). The amplified product was digested with restriction enzymes *HindIII* (New England Biolabs, Ipswich, US) and *XbaI* (Elpis-Biotech, Daejeon, Korea), and ligated into the same restriction sites of pcDNA3.1+ (Invitrogen, Grand Island, CA) using T4 DNA ligase (Elpis-Biotech) to generate a pcDNA-TYR construct. The construct was propagated in the *E. coli* strain DH5α (Elpis-Biotech) and purified using Endo-free Maxi-Prep DNA purification kits (Qiagen, Valencia, CA). The purified plasmid DNA was linearized by digesting it with *MfeI* followed by purification with a gel-purification kit (SolGent, Daejeon, Korea).

GENERATION OF A STABLE CELL LINE THAT EXPRESSES HUMAN TYR

HEK293 cells were transfected with the linearized plasmid construct using Lipofectamine 2000 (Invitrogen) as previously described (14). Briefly, cells at about 20% confluency in a 100-mm culture dish were washed with PBS and treated with a mixture of 5 μg of linearized DNA and 10 μl of Lipofectamine in 5 ml of Opti-MEM (Invitrogen) for 18 h. The transfected cells were cultured in a normal growth medium for one day. Then the cells were subcultured in a medium supplemented with 1 mg ml^{-1} of G418 (Geneticin®, Invitrogen). The culture medium was changed every four days, and after a total of three weeks, the culture dishes were examined for surviving colonies. Healthy-looking colonies that seemed to have dark granules were harvested and distributed to 96-well plates at a density of one cell per well. Among the several colonies that grew rapidly, the most dark-colored colony was chosen and replated on 96-well plates for the second-round selection. The most rapidly proliferating colony was expanded further into a cell line named HEK293-TYR.

IN VITRO ASSAY FOR TYR ACTIVITY USING CELL-FREE EXTRACTS

Cells were homogenized in an ice-cold lysis buffer (10 mM Tris-Cl, pH 7.4; 120 mM NaCl; 25 mM KCl; 2.0 mM EGTA; 1.0 mM EDTA; and 0.5% Triton X-100 and

protease inhibitor cocktail). The cell homogenates were centrifuged at 13,000 × g for 15 min at 4°C to obtain cell-free extracts. The *in vitro* TYR assay was done on a 96-well microplate with the reaction mixture (200 μl) containing 100 mM sodium phosphate buffer (pH 6.8), cell-free extracts (40 μg protein), 0.5 mM L-tyrosine, and 1 μM DOPA. When indicated, 1~1000 μM of test samples were included in the reaction mixture. The reaction mixture was incubated for 120 min at 37°C, and DOPA chrome formation was estimated at 490 nm by a BioRad Model 680 microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA).

WESTERN BLOTTING

Cell-free extracts containing 40 μg of protein were diluted in Laemmli buffer containing 2% SDS and 1.2% dithiothreitol, and heat-denatured at 95°C for five minutes. Proteins were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and the separated proteins were transferred to a polyvinylidene fluoride membrane (Pall Corporation, Port Washington, NY). The membrane was incubated with a primary antibody overnight at 4°C, and then with a secondary antibody conjugated with horseradish peroxidase for one hour at room temperature. The bands were detected using Enhanced Peroxidase Detection reagents (Elpis-Biotech) according to the manufacturer's instructions and subjected to densitometry analysis. Primary antibodies for TYR and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

ASSAY OF THE TYR-DEPENDENT MELANIN SYNTHESIS IN CELLS

Cells were cultured with or without addition of 1.0 mM L-tyrosine. The medium was replaced every other day and the cells were cultured for a total of six days. In some experiments, HEMs were pretreated with 100 μM of TYR inhibitors 60 min prior to the addition of 1.0 mM L-tyrosine. Accumulation of melanin-like materials inside of cells was highlighted by Fontana–Masson staining (15). Cells were fixed in 4% *p*-formaldehyde for 10 min at room temperature and stained for melanin using a Fontana–Masson staining kit from American Master*Tech Scientific, Inc. (Lodi, CA). Cells were stained with ammoniacal silver solution for 30 min at 60°C, followed by incubation in 0.1% gold chloride solution and then in 5% sodium thiosulfate solution. Cell morphology and pigmentation were examined under a phase-contrast microscope (Eclipse TE2000U, Nikon Instruments Inc., Melville, NY). Intracellular melanin was extracted with 100 mM NaOH at 60°C for 60 min. The optical density at 490 nm for melanin was normalized for the protein content determined by Bio-Rad DC assay. Cell viability was assayed using 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (16,17).

RESULTS AND DISCUSSION

In order to establish a cell line that grows rapidly and expresses human TYR constitutively, HEK293 cells were transfected with a human TYR construct and cloned based on

their ability to accumulate melanin-like materials inside the cells. The established cell line, HEK293-TYR, was expanded and examined for the ability to express active TYR and synthesize melanin.

The expression of active TYR was compared among HEMs, control HEK293 cells, and HEK293-TYR cells by TYR activity assay *in vitro* using cell-free extracts. The results showed that the specific TYR activity of HEK293-TYR cell extracts was close to that of HEM extracts, whereas no significant activity was seen with the extracts of the control HEK293 cells (Figure 1A). The cell-free extracts of HEMs, control HEK293 cells, and HEK293-TYR cells were also subjected to Western blot for comparison of TYR protein expression. The results confirmed that the TYR protein expression level relative to that of GAPDH, a housekeeping protein, in HEK293-TYR cells was similar to that in HEMs, whereas no TYR protein was detected in the control HEK293 cells (Figure 1B). The minor bands detected in the HEK293-TYR cells may be TYR protein that was not fully maturated via glycosylation (18). This result is consistent with the results of the TYR activity assay above.

The potential ability of HEK293-TYR cells to produce melanin-like materials was examined in comparison with HEMs and control HEK293 cells. These cells were treated

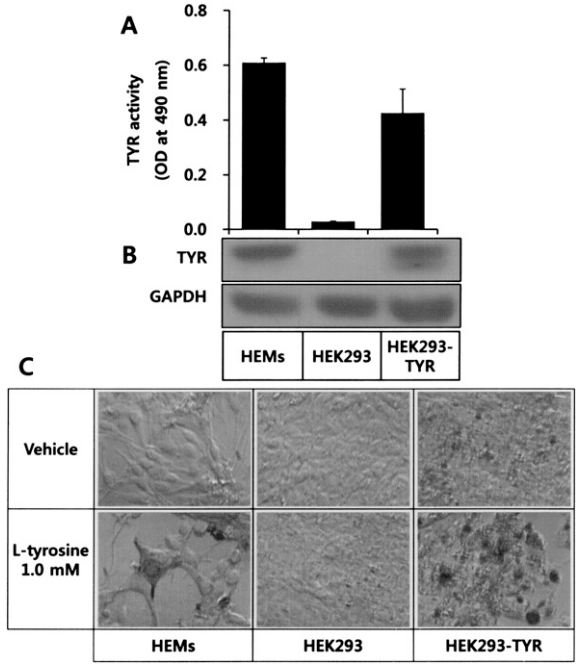

Figure 1. Generation and analysis of the HEK293-TYR cell line. HEK293 cells were transfected with a human TYR construct and subjected to cloning to establish a HEK293-TYR cell line as described in Materials and Methods. The expression of active TYR in HEMs, control HEK293 cells, and HEK293-TYR cells was compared by *in vitro* TYR activity assay (A) and Western blotting for TYR and GAPDH (B), using the cell-free extracts of those cells. Data represent means ± SE (n=3). Blots shown are representative of three independent studies. The melanogenic properties of HEMs, control HEK293 cells, and HEK293-TYR cells were compared by Fontana-Masson staining for melanin after incubation of those cells with or without 1.0 mM L-tyrosine for six days (C).

with 1.0 mM L-tyrosine or vehicle for six days with the medium changed every other day and subjected to Fontana-Masson staining of the melanin. As shown in Figure 1B, dark melanin was clearly observed in the HEMs and HEK293-TYR cells but not in the control HEK293 cells, indicating that HEK293-TYR cells acquired the ability to synthesize melanin.

Previous studies have revealed the anti-melanogenic properties of *p*-coumaric acid, a very common secondary metabolite of plants, enough to attract special attention as a potential hypopigmenting agent (19–21). The inhibitory effect of *p*-coumaric acid against mushroom TYR was determined to be only comparable to kojic acid, but its inhibitory effects against the human enzyme was ~100 times stronger than that of kojic acid (11). Because *p*-coumaric acid has a chemical structure very similar to that of L-tyrosine, the substrate of TYR, this compound might have acted as a pseudosubstrate that binds to the active site of the enzyme but does not undergo any further reaction. In this regard, other similar compounds were assumed to have an effect on TYR activity.

Taking advantage of the HEK293-TYR cell-free extracts, the effects of various phenylpropanoids against human TYR activity were examined *in vitro*. A test compound was included in the reaction mixture at different concentrations (0 ~ 1000 μM). The reaction was run with and without substrates to correct for any non-specific absorbance of a test compound. As shown in Figure 2, the strongest inhibition of TYR activity was observed with *p*-coumaric acid ($IC_{50,}$ 3 μM), followed by 3-(4-hydroxyphenyl)propionic acid

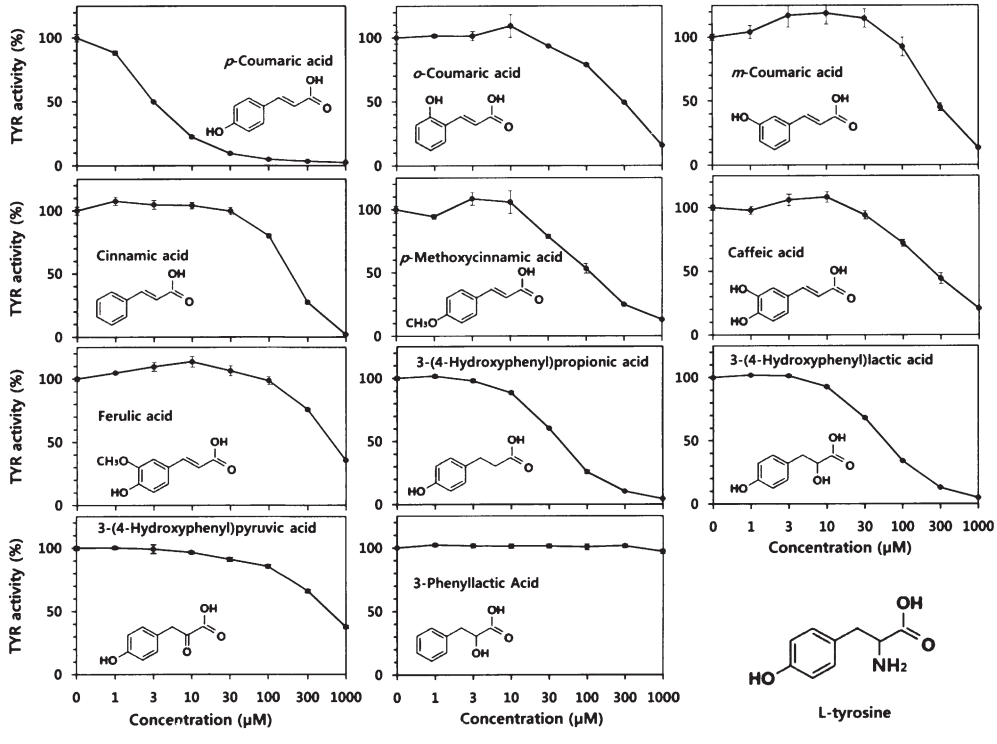

Figure 2. Use of the HEK293-TYR cell-free extracts in screening assay for TYR inhibitors. The effects of various phenylpropanoids aganist TYR activity were determined *in vitro* using the HEK293-TYR cell-free extracts. The chemical structures of phenylpropanoids and L-tyrosine are shown. Data represent means ± SE (n=3).

(50 μM), 3-(4-hydroxyphenyl)lactic acid (70 μM), *p*-methoxycinnamic acid (120 μM), cinnamic acid (200 μM), caffeic acid (250 μM), *m*-coumaric acid (270 μM), *o*-coumaric acid (300 μM), 3-(4-hydroxyphenyl)pyruvic acid (700 μM), ferulic acid (750 μM), and 3-phenyllactic acid (>1000 μM). The results indicate that the single phenolic hydroxyl group at the *para* position and the double bond on the side chain are critical for TYR enzyme inhibition. Additional hydroxyl or methoxy groups on the phenyl moiety and the hydroxyl and carbonyl groups on the side chain appear to have negative effects on TYR enzyme inhibition. The results support that *p*-coumaric acid has an optimal structure for the inhibition of TYR activity. As expected, the IC_{50} value of *p*-coumaric acid against TYR activity in HEK293 cell extracts was identical to the value previously determined using HEM extracts (3 μM) and very different from the value against mushroom TYR (300 μM) (11).

Because *p*-coumaric acid, 3-(4-hydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)lactic acid appeared to be strong inhibitors of human TYR, their effects on cellular melanogenesis were compared in HEMs. In this experiment, cells were pretreated with a test compound and then treated with L-tyrosine to stimulate melanogenesis (22). As shown in Figure 3A,B, treatment of HEMs with L-tyrosine decreased cell viability by 7.5% and increased intracellular melanin content by 68%. Among the three test compounds, *p*-coumaric acid inhibited the cellular melanin synthesis most effectively without significant cytotoxicity (Figure 3A,B). The other two compounds had no significant effects on cellular melanogenesis. Thus the inhibitory effects of these phenylpropanoids on cellular melanogenesis correlate well with their inhibitory effects on TYR activity *in vitro*, supporting the usefulness of the TYR inhibitor screening method developed in this study. These results also support a great potential of

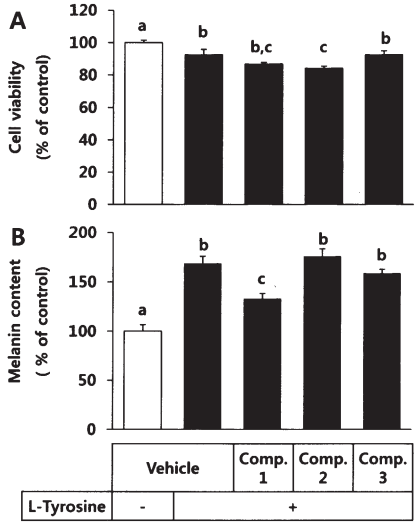

Figure 3. Effects of *p*-coumaric acid, 3-(4-hydroxyphenyl)propionic acid and 3-(4-hydroxyphenyl)lactic acid on melanin synthesis in HEMs. HEMs were pretreated with the vehicle or *p*-coumaric acid (Comp. 1), 3-(4-hydroxyphenyl)propionic acid (Comp. 2), or 3-(4-hydroxyphenyl)lactic acid (Comp. 3) at 100 μM and then stimulated with 1.0 mM L-tyrosine. Cell viability (A) and intracellular melanin content (B) are presented as percent of vehicle control without L-tyrosine treatment (means ± SE, n=4). Data not sharing the same letters are significantly different from each other.

p-coumaric acid as a hypopigmenting agent whose *in vivo* efficacy was demonstrated in a human study (23).

CONCLUSIONS

Although non-melanocytic cells such as fibroblasts and Chinese hamster-human hybrid ovary cells had been transfected with a human TYR construct for other purposes in previous studies (12,24), the present study demonstrated for the first time that cell-free extracts of the established cell line, HEK293-TYR, may be conveniently used in the screening of TYR inhibitors as potential hypopigmenting agents. The rapid proliferation of this cell line in a relatively inexpensive culture medium should be a big advantage over HEMs. Thus, this cell line can be a good source of active human TYR enzyme and should help in the screening of hypopigmenting agents for cosmetic purposes.

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