Effects of Banha Extract on the Melanin Biosynthesis and Tyrosinase mRNA Level in B16 Mouse Melanoma Cells

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반하 추출물이 B-16 마우스 흑색종 세포의 멜라닌 생성과 타이로시네이즈 mRNA 양에 미치는 영향

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ABSTRACT

Melanin pigmentation in human skin is a major defense mechanism against ultraviolet light of the sun. Tyrosinase(EC 1.14.18.1) plays a key role in the biosynthesis of melanin. This is why much researches have been focused on its regulation in controlling the epidermal melanization. We have found that the water-extract of Banha(Pinelliae ternate B.), an oriental medicinal plant, has no tyrosinase inhibitory activity, but does inhibit the melanin biosynthesis in B16 mouse melanoma cells. We also found that Banha extract lowers the tyrosinase activity in cultured cells. To elucidate the action mechanism of Banha extract we have investigated its effect on the tyrosinase mRNA level using reverse transcription-polymerase chain reaction(RT-PCR) technique. It was revealed that Banha extract reduced the tyrosinase mRNA level in a dose dependent manner; when B16 mouse melanoma cells were cultured with 2mg/ml and 5mg/ml of Banha extract, there were 20% and 44% decrease in tyrosinase mRNA level, respectively. These data suggest that the Banha extract exerts its melanogenic inhibitory effect through the transcriptional regulation of tyrosinase mRNA.

INTRODUCTION

Melanin pigmentation in human skin is a major defense mechanism against ultraviolet light of the sun, but abnormal pigmentation such as freckles, chloasma (liver spot, melasma) could be a serious aesthetic problem. Tyrosinase and other enzymes, such as tyrosinase related protein 1(TRP-1), tyrosinase related protein 2(TRP-2, Dopachrome tautomerase), are responsible for the biosynthesis of melanin. Among these enzymes, tyrosinase plays the most important role in melanin formation. Tyrosinase(EC 1.14.18.1) is a copper containing enzyme and exclusively expressed in melanocytes, melanin producing cells in epidermis. Tyrosinase itself could produce melanin pigments without any help of other melanogenic enzymes.[1] In fact, fibroblast cells, transfected with tyrosinase cDNA, produced melanin in their lysosome-like structures.[2-4] It is not fully understood that how the expression of tyrosinase is regulated. Lots of studies searching for melanogenic inhibitory compounds are currently undergoing to prevent or to cure these hyperpigmentary disorders. Nearly all studies are mainly concentrated on searching for the materials that have inhibitory activities on tyrosinase, a key enzyme for melanin biosynthesis.

Here we report the Inhibitory effect of Banha extract (*Pinelliae ternate* B.) on the melanin biosynthesis of B16 mouse melanoma cells and that its effect is exerted by transcriptional regulation of tyrosinase gene.

MATERIALS AND METHODS

Preparation of Banha extract

Banha was purchased from oriental pharmacy. Banha was extracted with 20% aqueous ethanol, filtered and dried under reduced pressure. The extract was dissolved in distilled water or in culture medium and then used for each experiment.

Cell culture

B16 mouse melanoma cells were cultured in DMEM supplemented with 10% fetal calf serum in humidified incubator at 37℃ under 5% CO_2.

Effect of Banha extract on melanization of B16 mouse melanoma cells

Cells were seeded into 60㎜ petridish at a density of $5x10^5$ cells per dish. After cells were attached, medium was replaced with fresh medium containing various concentrations of Banha extract. Then cells were cultured for 2 days and the medium was replaced with fresh medium with Banha, further incubated for a day. Then cells were harvested with cell scrapper, counted with hemacytometer and collected by centrifugation. Melanin was extracted and measured according to the method of Lotan with some modifications.[5] Briefly, cell pellets were resuspended in 1㎖ of distilled water and freezed at -20℃ and thawed at 37℃. This freezing-thawing process was performed for total of three times. Perchlonic acid was added to the cell suspensions at a final concentration of 0.5N. The tubes were set on ice for 10 min and centrifuged at 15,000g for 5 min. The pellets were extracted with 0.5N perchloric acid for 2 times, with cold ethanol/ether (3:1) for 2 times, and finally with ether. The resulting pellets were dried in air and 1㎖ of 1N NaOH was added to each tube. The tubes were incubated in a boiling water bath for 10 min to dissolve the pellets. Melanin contents were measured by reading the absorbances at 400 ㎚ and expressed as $A_{400}/10^6$ cells.

Effect of Banha extract on the activity of tyrosinase in B16 melanoma cells

B16 cells were cultured for 3 days with various concentrations of Banha extract. The cells(about $2x10^7$ cells) were collected by centrifugation and washed with ice-cold PBS, resuspended in 1㎖ of homogenization buffer (50 mM sodium phosphate, pH 6.8, 1% Triton X-100, 2mM PMSF) and homogenized by ultrasonication. The cell lysate was centrifuged at 15,000g for 20 min at 4℃ and resulting supernatants were used for tyrosinase assay and protein determination. Tyrosinase activity was measured as follows. The reaction mixture contained 50mM sodium phosphate (pH 6.8), 0.3㎖ of 1.5mM tyrosine, 30㎕ of 0.06mM Dopa and 0.3㎖ of cell lysate. The reaction mixtures were incubated at 37℃ for 30 min and absorbances at 475nm were measured. Tyrosinase activity was expressed as A475/㎎ protein. Protein quantitation was performed using Bio-Rad protein assay kit, following the supplier's instruction.

In situ tyrosinase assay

Tyrosinase activity in living cells was assessed according to the method of Pomerantz with slight modifications.[6] $5x10^5$ cells were seeded into 60㎜ petridish and cultured for 2 days and the medium was replaced with fresh medium containing 1mCi/㎖ of ^3H tyrosine(Amersham, UK) and further incubated for 24 hr. To measure ^3H_2O release, 1㎖ of culture medium was mixed with activated charcoal and incubated with agitation for 30 min at RT. The mixture was centrifuged at 14,000g for 10 min, and 500㎕ of supernatant was taken into liquid scintillation vial, mixed with scintillation cocktail, and the radio activity was determined by LS 6500 scintillation system (Beckman, USA).

RT-PCR analysis of tyrosinase mRNA

mRNAs from cultured cells were isolated using Promega polytract system 1000 mRNA isolation kit according to the supplier's instruction. Finally, 80~90㎍ of mRNA was isolated from 10^8 cells. Primers used for RT-PCR analysis of tyrosinase mRNA were as follows; 5'primer; 5'GACCTCAGTTCCCCTTCAAA3'(197-216 from ATG start codon), 3'primer; 5' TCTCATCCCCAGTTAGTTCT 3'(669-688 from ATG start codon). These primers were synthesized by Bioneer co., Korea. For cDNA synthesis, 20ng of mRNA was reverse transcribed in 20㎕ of reaction mixture containing 2㎕ of 10x reverse transcription buffer, 2㎕ of 10mM $MnCl_2$, 0.2mM of each dNTPs, 50 pmole of 3' primer and 5 U of rTth DNA polymerase(Perkin Elmer). This enzyme reverse transcribes RNA in the presence of Mn^{++}. Reverse transcription reaction mixture was incubated at 94℃ for 1 min, at 55℃ for 30 seconds and at 72℃ for 10 min. For PCR amplification of cDNA, 80㎕ of PCR mixture containing 8㎕ of 10x chelating buffer, 1mM $MgCl_2$ and 50 pmole of 5' primer was added to RT reaction mixture. DNA amplification was performed using a Perkin Elmer Gene Amp PCR system 2400 thermal cycler. The PCR cycle conditions were melting for 30 seconds at 94℃, annealing for 30 seconds at 55℃, extension for 1 min at 72℃. Reaction mixtures were cycled for 28 cycles. PCR products (492bp) were resolved on 2% agarose gel and visualized by ethidium bromide staining, photographed and quantitated by image analysis (imagemaster 1D elite, Pharmacia). As internal controls, mouse β-actin mRNAs were also amplified using mouse β-actin amplimer set(Clontech, USA) following the manufacturers instruction.

RESULTS AND DISCUSSIONS

Effect of Banha extract on melanization of B-16 melanoma cells

Since Banha extract has no tyrosinase inhibitory activity(data not shown), we investigated the effect of Banha extract on the melanin content of B-16 melanoma cells(Fig. 1). When cultured with 1 ㎎/㎖, 2㎎/㎖ and 5㎎/㎖ of Banha extract, there were 36%, 77% and 90% decrease in melanin contents of B-16 melanoma cells, respectively. These are very significant decrease in melanin contents when compared with other melanogenic inhibitors. These data indicate that Banha extract, though it has no tyrosinase inhitory activity, has whitening effect and its effect is exerted by some other mechanisms.

Effect of Banha extract on tyrosinase activity in B16 melanoma cells

To determine the effect of Banha extract on tyrosinase activity in living cells we performed in situ tyrosinase assay. Banha extract dramatically decreases the in situ tyrosinase activity of B16 melanoma cells (Fig. 2, A). Treatment of Banha extract also reduced the specific activity of tyrosinase protein extract from B16 melanoma cells(Fig. 2, B), consistent with the result of in situ tyrosinase assay. When B16 cells were cultured with 1 ㎎/㎖, 2㎎/㎖ and 5㎎/㎖ of Banha extract, there were 53%, 68% and 87% decreases in tyrosinase activity, respectively. These data mean that Banha extract decreases the total tyrosinase activity, and exerts its effect in some novel manner.

RT-PCR analysis of tyrosinase mRNA

To elucidate the mechanism by which Banha extract exerts its effect, we performed RT-PCR analysis. Banha extract did decrease the amount of tryosinase mRNA in a dose-dependent manner(Fig. 3).

In the present study, we have demonstrared that extract of Banha, though it has no detectable tyrosinase inhibitory activity, had a depigmenting effect on cultured B16 mouse melanoma cells and this depigmenting effect was exerted by downregulation of tyrosinase mRNA. The exact mechanism by which the tyrosinase mRNA level is downregulated is to be elucidated.

CONCLUSIONS

1. Extract of Banha(*Pinelliae ternate* B.), an oriental medicinal plant, has potent inhibitory effect on melanin biosynthesis in B16 mouse melanoma cells.
2. The melanogenic inhibitory effect of Banha extract is exerted by downregulation of tyrosinase mRNA.

요약

피부 내에서의 멜라닌 색소의 생성은 태양광에 존재하는 유해한 자외선으로부터 생체를 보호하는 중요한 방어 수단으로 여겨지고 있다. 타이로시네이즈(tyrosinase, EC 1.14.18.1)라는 효소가 이러한 멜라닌의 합성 과정에 있어서 매우 중요한 역할을 수행한다. 이러한 이유로 피부의 색소생성을 조절하는 많은 연구의 대부분이 타이로시네이즈의 효소적 조절에 초점이 맞추어져 왔다. 본 연구자들은 전통 한약재인 반하(Pinelliae ternate B.)의 물 추출물이 비록 타이로시네이즈에 대한 저해 작용은 없으나 배양된 B-16 마우스 흑색종 세포의 멜라닌 생성을 억제한다는 것을 발견하였다. 본 연구자들은 또한 반하 추출물이 배양된 세포의 타이로시네이즈 효소 활성을 낮춘다는 사실도 발견하였다. 이러한 반하 추출물의 작용 기작을 밝혀내기 위하여 반하 추출물이 B-16 마우스 흑색종 세포의 타이로시네이즈 mRNA 양에 미치는 영향에 대하여 reverse transcription-polymerase chain reaction(RT-PCR) 기법을 이용, 연구를 수행 하였다. 연구 결과 반하추출물은 타이로시네이즈 mRNA의 양을 용량 의존적인 경향으로 감소시킴이 밝혀졌다. 즉, 반하추출물을 2㎎/㎖ 및 5㎎/㎖ 처리시 각각 20%와 44%의 mRNA 양의 감소가 관찰 되었다. 이러한 결과는 반하 추출물이 타이로시네이즈 mRNA의 전사 조절을 통하여 그 미백효과를 발휘한다는 것을 의미한다.

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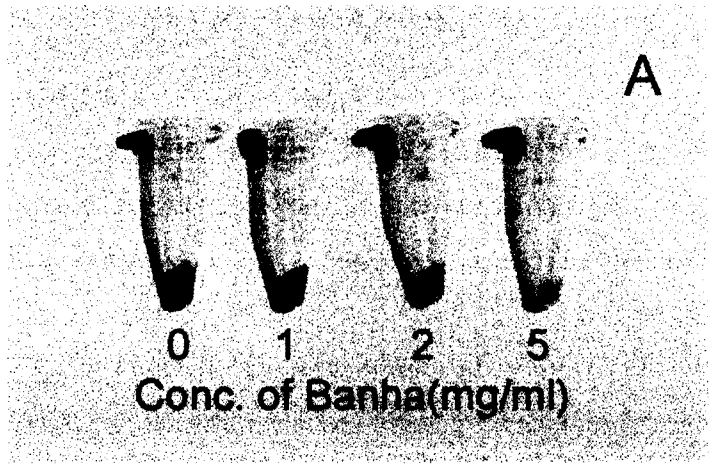


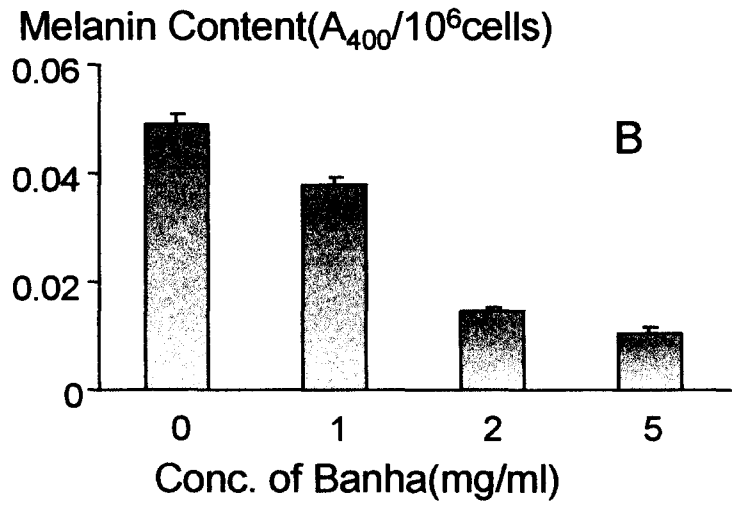
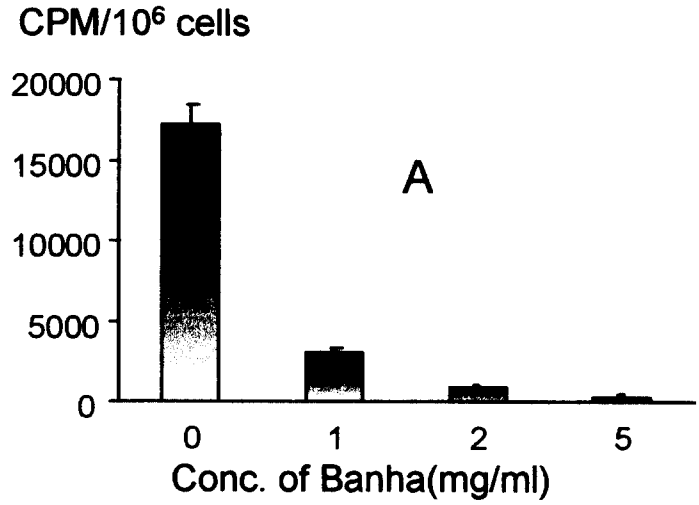


Fig. 1. Effects of Banha extract on pigmentation(A) and melanin contents(B) of B16 mouse melanoma cells(n=3).



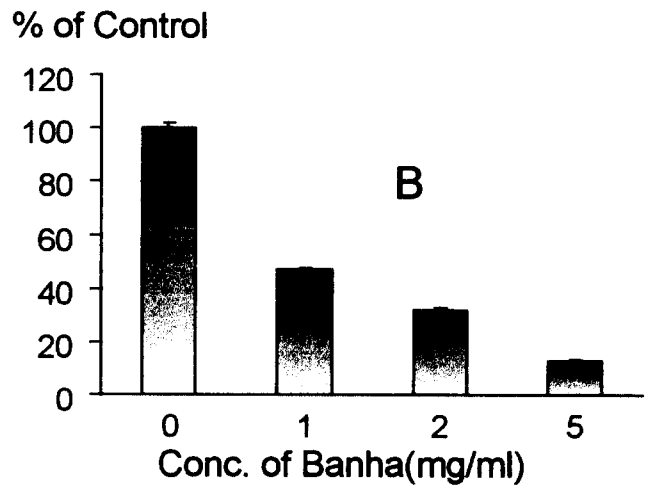


Fig. 2. Effects of Banha extract on tyrosinase activity of B16 melanoma cells in situ(A) and tyrosinase activity in B16 melanoma cells(B);n=3

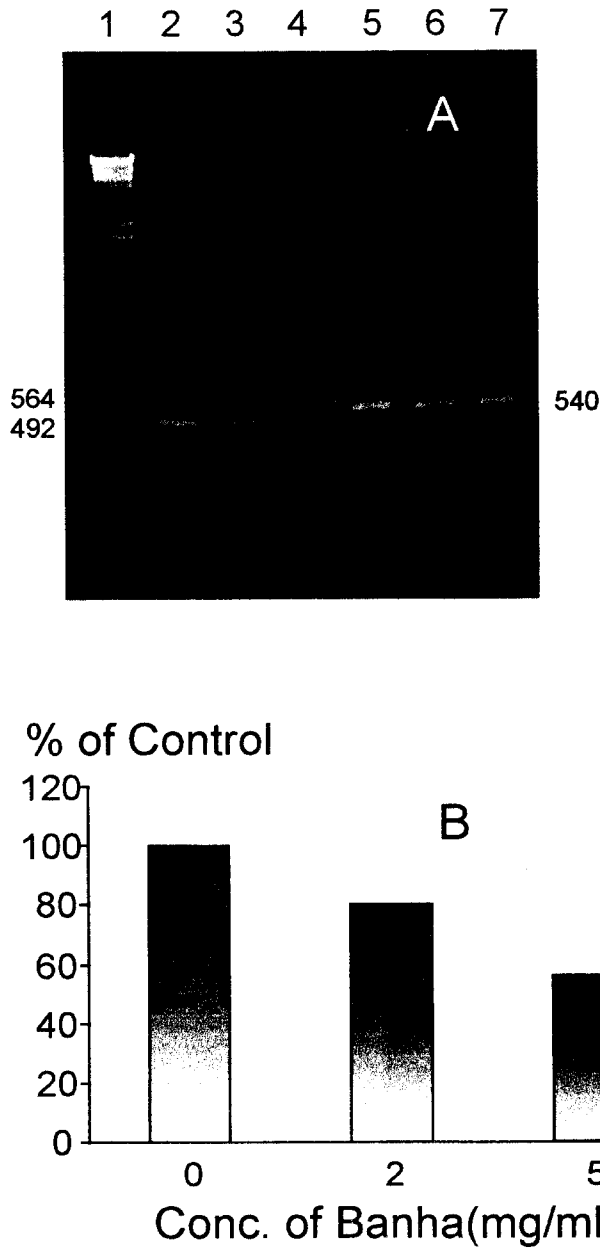


Fig.3.Banha reduces tyrosinase mRNA level in B16 mouse melanoma cells. (A) Tyrosinase and mouse β-actin PCR products were loaded on 2% agarose gel, stained with EtBr. Lane1: size marker, lane2, 3, 4: PCR product of tyrosinase, lane5, 6, 7: PCR product of internal control(β -actin, 540 bp), lane2, 5: mRNA from control culture, lane3, 6: mRNA from 2㎎/㎖ of Banha treated cuture, lane4, 7: mRNA from control culture, lane3, 6: mRNA from 2㎎/㎖ of Banha treated cuture. (B) Each band was quantitated by image analysis using β -actin bands as references.