The Effects of Whitening Components on Human Melanocytes in vitro

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Abstract

To indentify inhibitors of melanogenesis, we compared the effects of 5 compounds(arbutin, ascorbic acid, azelaic acid, kojic acid, and tranexamic acid) on mushroom tyrosinase, human melanocytic tyrosinase activity and melanin content. The cytotoxicity of the components were also tested on cultured human melanocytes.

Kojic acid showed marked inhibitory effect both on mushroom and human tyrosinase activity. This action of kojic acid is stronger than that of ascorbic acid. Arbutin inhibited human tyrosinase activity of cultured melanocytes although it had slightly inhibitory effect on mushroom tyrosinase activity. Azelaic acid had no effect on human tyrosinase activity. Melanin production was inhibited significantly by kojic acid and tranexamic acid. MTT assay showed that all of the compounds were non-cytotoxic to melanocytes at the concentrations tested.

These results suggest that the effects of kojic acid on cultured melanocytes involve inhibition of tyrosinase activity and melanogenesis without affecting the cell number.

Introduction

Melanin is the main pigment found in skin, hair and eyes. It is synthesized enzymatically from tyrosine within melanosomes, which are subsequently transferred to epidermal keratinocytes. Tyrosinase is a multifunctional enzyme responsible for this melanin biosynthesis(1-3).

Hyperpigmentaion is usually asymptomatic and of no medical consequence. However, this condition, particularly on the face, can be a source of cosmetic disability and mental distress and thus may require treatment.

Over several decades, considerable data have been gathered regarding depigmenting chemical agents. Only recently, however, have their action mechanisms been investigated.

The objevtive of this study was to determine the effect of kojic acid, ascorbic acid, arbutin, azelaic acid, and tranexamic acid on tyrosinase activity, melanogenesis and proliferation of cultured human melanocytes.

Materials & Methods

Reagents

Arbutin, kojic acid, ascorbic acid, azelaic acid, tranexamic acid were purchased from Sigma (St. Louis, MO). All the final concentration of reagents tested was 1mM.

Melanocyte growth media

Growth medium used for cultures of melanocytes was MCDB supplemented with 4% fetal calf serum(FCS), 5 nM 12-0-tetradecanoylphorbol-13-acetate (TPA) (Sigma), 0.6 ng/ml basic fibroblast growth factor(bFGF) (Calbiochem, San Diego, CA), 1ng/ml vitamin E, 1ug/ml transferrin, 5ug/ml insulin and antibiotics.

Cell culture

Human newborn foreskin melanocytes were cultured either by a modification of the method of Eisinger and Marko(4) or a modification of the Halaban and Alfano(5). Briefly, Neonatal foreskins obtained from routine circumcisions were used to culture human melanocytes.They were cleaned of subcutaneous tissue, cut into small pieces (4x4mm), rinsed in calcium-, magnesium-free phosphate-buffered saline (PBS, pH 6.8), and incubated in 0.25% trypsin solution at 4°C for 12-16 hr. And then, epidermal sheets were mechanically separated from the dermis using fine forceps. Isolated epidermal sheets were pooled in growth media and single-cell suspensions were prepared by vortex. The suspension was plated to T-25 flasks in growth media and incubated at 37°C in a humidified atmosphere composed of 5% CO2 . Medium changes were performed twice a week.

Serial subculture

Third passage melanocytes were treated with 0.05% trypsin-0.02% EDTA solution at 37°C for 2-3 min, harvested with MCDB 153 containing 10% FCS, and resuspended in MCDB 153 containing 10% fetal calf serum. The resulting cells were counted in a hemocytometer chamber at a magnification of x 200. The melanocytes were placed in 96-well plates at a density of 20,000 cell/well. Each agent was added to three wells, and culture was performed at 37°C for 3 days.

Cell viability assay

Melanocytes were seeded at a density of 2 x 10^4 cells / 150 µl / well in various culture media The proliferation of cultured melanocytes was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide , Sigma) assay, as described (6). Briefly, 15 µl of MTT solution (5mg/ml in PBS) was added to the medium and cells were incubated for 3h at 37°C. The medium was gently removed from each well and 150 µl of DMSO (dimethyl sulfoxide) was added. After 30 minutes, the plates were read on a microelisa reader, using a test wavelength of 540 nm, a reference wavelength of 650 nm.

Effect on mushroom tyrosinase actvity

Each assay contained 200 ul of freshly prepared L-dopa (1mM) in PBS (pH 6.8), variable concentrations of test reagents, and mushroom tyrosinase (25 U/ml). After incubation of the plates at 37°C for 10, 30, and 60 min, absorbance was measured at 490 nm in a Model-3550 ELISA Reader (Bio-Rad Lab, Richmond, CA).

Effect on human tyrosinase activiy

Cellular tyrosinase activity was assayed by the method of Maeda *et al* (7), which we modified. Briefly, human melanocytes(100,000cells/well) cultured in 6-well plates were cultured with each agent(1mM) at 37°C for 3 days. The melanocytes were washed with PBS and lysed with 90 µl of 1% Triton-X. Then 10 µl of L-dopa (10mM) was added to the wells. After incubation at 37°C for 60 min, the plates were read on a microelisa reader at 490 nm.

Melanin assay

Melanin content was determined according to the method of Oikawa *et al* (11), which we modified. Briefly, human melanocytes(100,000cells/well) cultured in 6-well plates were cultured with each agent(1mM) at 37°C for 3 days. The melanin content of melanocytes was determined by removing the medium and washing cells with PBS. After washing, the cells were lysed by the addition of 1.0ml of 1 N NaOH and repeated manual pipeting. The crude cell extracts were assayed using a spectrophotometer at 405 nm to determine melanin content. Results are expressed as ug of melanin per 1 x 10^6 cells. A standard curve was obtained with synthetic melanin(Sigma Chemical Co., St. Louis, MO).

RESULTS

1. Effect on mushroom tyrosinase activity (Fig. 1)

The inhibitory effect of arbutin, kojic acid, ascorbic acid, azelaic acid, and tranexamic acid on mushroom tyrosinase was compared. Kojic acid showed marked inhibitory effects in a dose-dependent and a time-dependent manner. This action of kojic acid is stronger than that of ascorbic acid or azelaic acid. Ascorbic acid or azelaic acid caused no significant changes in mushroom tyrosinase activity between 1uM and 100 uM, but markedly inhibited it at 1mM. Arbutin slightly inhibits the mushroom tyrosinase activity, but tranexamic acid did not show that.

2. Effect on human tyrosinase activity (Fig. 2)

Kojic acid, ascorbic acid, and arbutin significantly inhibited dopaoxidase activity of human tyrosinase. Inhibitory effects of azelaic acid and tranexamic acid were minimal or absent.

3. Melanin assay (Table 1)

Arbutin, kojic acid, and tranexamic acid inhibited melanin formation, whereas ascorbic acid and azelaic acid rather increased melanin formation of cultured human melanocytes at the concentration of 1mM.

4. Effect on proliferation of melanocytes (Fig. 3)

The effects of depigmenting components on the proliferation of cultured melanocytes was determined with MTT assay. MTT assay showed that all of the compounds were non-cytotoxic to melanocytes at the concentrations tested (1mM).

The summarized result was shown in Table 2.

Discussion

Tyrosinase is a copper-containing monophenol monooxygenase and is well known as the critical enzyme in the pathway of melanogenesis(1-3). Several chemicals including arbutin, kojic acid, azelaic acid, ascorbic acid and tranexamic acid have been suggested as a depigmenting compounds. It is still uncertain how tyrosinase and the melanin pathway are regulated by various chemicals known as whitening agents.

We have shown that kojic acid has marked inhibitory effect both on mushroom and human tyrosinase activity. This chemical also inhibited melanin formation of cultured human melanocytes. These finding correlate well with those of Mishima et al(8) who have suggested that action mechanism of kojic acid is chelation of tyrosinase copper. The action of kojic acid is stronger than that of ascorbic acid. Azelaic acid had no effect on human tyrosinase activity in our study, although its inhibitory effect on tyrosinase has been suggested(9). Tranexamic acid inhibited melanin production without any change in tyrosinase activity. We have no idea about this finings but a likely possibility is trenexamic acid might inhibit melanin formation with a tyrosinase-independent manner, such as post-tyrosinase regulatory system. Arbutin inhibited human tyrosinase activity of cultured melanocytes although it did not have inhibitory effect on mushroom tyrosinase activity. There has been a suggestion that arbutin inhibited melanin formation (10), but we could not find the change. This discrepancy in the results may be due to the experimental conditions. Further experiments are needed to conform the exact action of arbutin.

MTT assay showed that all of the compounds were non-cytotoxic to melanocytes at the concentrations tested, indicating their depigmenting actions were not by destruction of melanocytes .

These results suggest that, among the chemicals tested, kojic acid has the strongest action of depigmentaion which involves inhibition of tyrosinase activity and melanogenesis without affecting the cell number.

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Table 1. Effects of depigmenting agents on melanin formation of cultured human melanocytes

	melanin content (μg /1x10^{6}cell)	inhibition (%)
control	5.057 ± 0.584	0.00 ± 11.5
AB	4.724 ± 0.084	6.59 ± 1.651
VC	5.391 ± 0.084	-6.59 ± 1.651
AA	5.558 ± 0.751	-9.91 ± 14.851
KA	4.140 ± 0.500	18.13 ± 9.887
TA	3.557 ± 0.583	29.66 ± 11.529

Cultured melanocytes were treated with each chemical compounds(1mM) for 3 days. Inhibition (%) = {1-(control value/test value)}x100 (%). AB : arbutin, VC : ascorbic acid, AA : azelaic acid, KA : kojic acid, TA : tranexamic acid.

Table 2. Effects of depigmenting agents on mushroom tyrosinase, human tyrosinase activity, melanin formation, and proliferation of cultured human melanocytes

	AB	VC	AA	KA	TA
mushroom tyrosinase	0	-	-	-	0
human tyrosinase	-	-	0	-	0
melanin formation	0	0	0	-	-
cell proliferation	0	0	0	0	0

+ : stimulation, - : inhibition, o : no change.
AB : arbutin, VC : ascorbic acid, AA : azelaic acid, KA : kojic acid, TA : tranexamic acid.

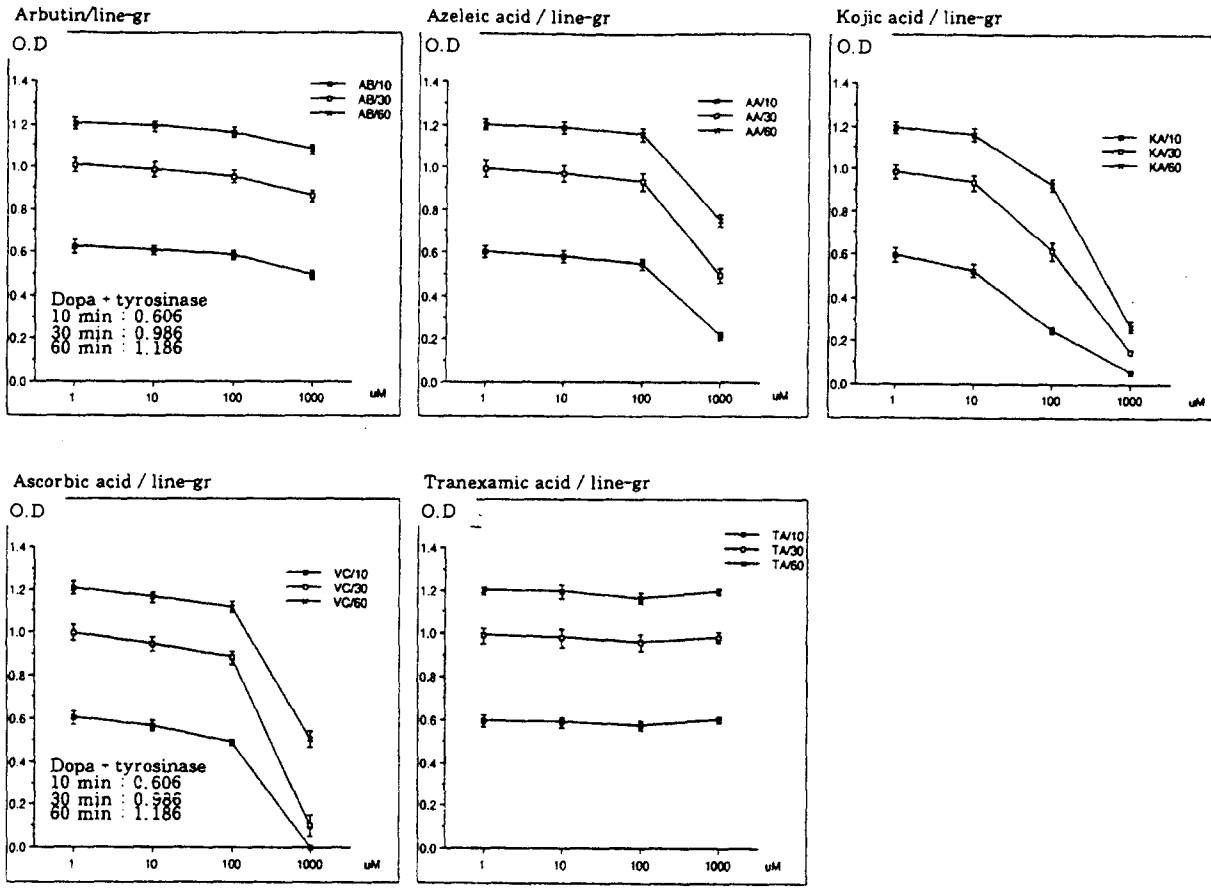

Fig. 1. Effects on mushroom tyrosinase activity. Muchroom tyrosinase (25 units/ml) and L-dopa (1mM) were reacted with each chemical compounds(concentration between 1 uM and 1 mM) for 10, 30, and 60 minutes. AB : arbutin, VC : ascorbic acid, AA : azelaic acid, KA : kojic acid, TA : tranexamic acid.

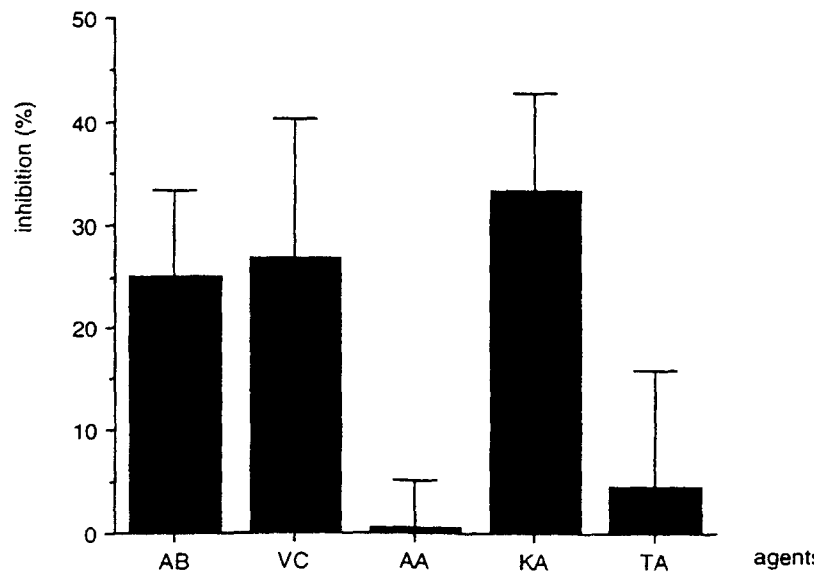

Fig. 2. Effects on human tyrosinase activity. Cultured melanocytes were treated with each chemical compounds(1mM) for 3 days. Inhibition (%) = {1-(control value/test value)}x100 (%). AB : arbutin, VC : ascorbic acid, AA : azelaic acid, KA : kojic acid, TA : tranexamic acid.

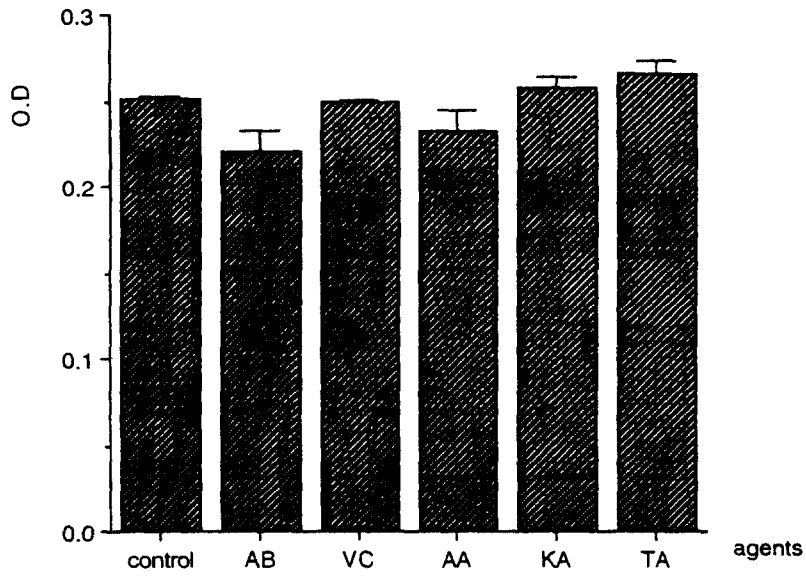

Fig. 3. Effects on proliferation of melanocytes. Cultured melanocytes were treated with each chemical compounds(1mM) for 3 days. AB : arbutin, VC : ascorbic acid, AA : azelaic acid, KA : kojic acid, TA : tranexamic acid.