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Original Article

DMEM enhances tyrosinase activity in B16 mouse melanoma cells and human melanocytes

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Abstract

Media components may affect the activities of cultured cells. In this study, tyrosinase activity was evaluated by using B16-F10 mouse melanoma cell lines (B16-F10) and primary human melanocytes cultured in different media. An optical density measurement and a L-dopa reaction assay were used as the determination of the tyrosinase activity. The study of B16-F10 found the optical density to be 2010, 2246 and 2961 in cells cultured in RPMI Medium 1640 (RPMI1640), Minimum Essential Medium (MEM) and Dulbecco's Modified Eagle Medium (DMEM), respectively. Moreover, compared to RPMI 1640 and MEM, DMEM showed the darkest color of melanin formation in culture media and in cells after the L-dopa reaction assay. Addition of kojic acid showed a significant inhibitory effect on tyrosinase activity in all media. Whereas MCDB153 showed no significant effect on human melanocytes, DMEM caused a dramatic increase in tyrosinase activity after 4 days of cultivation. Addition of kojic acid showed a significant tyrosinase inhibitory effect in DMEM only. Furthermore, an active ingredient in green tea, epigallocathechin gallate (EGCG) could inhibit tyrosinase activity in both B16-F10 and human melanocytes cultured in DMEM. In summary, these results suggest that DMEM is a suitable medium that provides high detection sensitivity in a tyrosinase inhibition assay.

Keywords: Tyrosinase activity, L-dopa reaction, B16-F10, melanocytes, EGCG

1. Introduction

Tyrosinase is a unique enzyme that is responsible for melanin synthesis in pigmented cells referred to as melanocytes. Melanogenesis, the pathway of melanin synthesis, begins with oxidation of the amino acid tyrosine to L-dopa and dopaquinone by the action of tyrosinase enzyme, and the reaction then progresses to melanin by auto-oxidation (Takeo, 1997). Since tyrosinase catalyzes a key rate-limiting step of melanogenesis, stimulation or inhibition of tyrosinase strongly affects the whole pigmentation process. Hence, measurement of tyrosinase activity is widely utilized to assess substances affecting pigmentation in cells (No *et al.*, 1999; Nagajima *et al.*, 1998; Wang *et al.*, 2006). Abnormal melanin production may result in melasma, freckles, and senile lentigenes and can be a serious aesthetic problem. Compounds such as kojic acid, arbutin and vitamin C have been used for de-pigmentation (Mishima *et al.*, 1994; Maeda and Fukuda, 1996; Shinomiya *et al.*, 1997), but the demand for alternative active compounds is rising and efficacy evaluation of these compounds is an important step in the development process.

Pigmented melanoma cell lines and melanocytes have often been used to assess the efficacy of melanogenic regulators *in vitro* (Wang *et al.*, 2006; Zye *et al.*, 2005). Among these, B16-F10 mouse melanoma cells have been used for investigation of the melanogenic effect of various compounds (Kosano *et al.*, 2000; Zhang *et al.*, 1992; Jacobsohn *et al.*,

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1988). A variety of media types has generally been used for culturing the B16-F10, including MEM (Kosano *et al.*, 2000), RPMI Medium 1640 (Kubo *et al.*, 2004) and DMEM (Terao *et al.*, 1992), and MCDB153 has been used for culturing human melanocytes (Kim *et al.*, 2002; Hedley *et al.*, 1998). However, it is likely that melanogenesis may be affected by media components, thus the purpose of this study was to evaluate the effects of different media on tyrosinase activity in order to identify the optimal medium for the further experiment. In addition, a second compound, epigallocathechin gallate (EGCG), was examined the tyrosinase inhibitory effect in both B16-F10 and primary human melanocytes.

2. Materials and Methods

2.1 Media, enzymes and chemicals

Dulbecco's Modified Eagle Medium (DMEM), Minimum Essential Medium (MEM), RPMI Medium 1640 (RPMI 1640), L-glutamine, human insulin, penicillin/streptomycin, transferrin, trypsin and phosphate-buffered saline (PBS) were purchased from Gibco Life Technologies, USA; fetal bovine serum (FBS) was purchased from Hyclone, USA; endothelin 1, MCDB 153, 3,4-dihydroxy-L-phenylalanine (L-dopa), epigallocatechin gallate (EGCG), alpha melanocyte-stimulating hormone (α-MSH), basic fibroblast growth factor (bFGF), kojic acid and formaldehyde were purchased from Sigma-Aldrich, USA; and Triton X-100 was supplied by Optimal Chemicals (Malaysia).

2.2 Cell cultures

2.2.1 B16-F10 mouse melanoma cells

Mouse melanoma cell lines (B16-F10, ATCC Cat. No. CRL-6475) were seeded at $5x10^4$ cells/35-mm dish in different media (DMEM, MEM or RPMI 1640) containing 5% FBS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 10nM α-MSH, and cultured at 37°C in a 5% CO_2 incubator. Kojic acid, a known tyrosinase inhibiter (Mishima *et al.*, 1994), was used as a positive control. After 3 days of initial cultivation, these cells were then cultivated for four days in a test medium: DMEM, MEM, RPMI 1640, DMEM+kojic acid, MEM+kojic acid, RPMI 1640+kojic acid, or DMEM+EGCG. The concentrations of kojic acid and EGCG were 300 and 5 μg/ml, respectively. Media were replaced with fresh test media every 2 days during cultivation. Each test medium was placed in dishes without cells as a control for color development. Throughout the culture period, the growth of cells was measured daily by cell counting. Briefly, cells were trypsinized and centrifuged at 500 g for 5 minutes. The pellets were resuspended in medium, and a portion of the cell suspension was added to an equal volume of 0.08% trypan blue to distinguish live and dead cells. Live cells were counted using a hemacytometer under a light microscope. Cellular tyrosinase activity was evaluated daily (day 4 to 7) by optical density (OD) measurement of melanin formation and by the L-dopa reaction on day 7.

2.2.2 Human melanocytes

The source of tissue for the melanocyte culture was discarded neonatal foreskins obtained from routine circumcision (approved by the ethical committee from the Faculty of Pharmaceutical Sciences, Chulalongkorn University). The samples were transferred to a sterile tube with 0.5% dispase in PBS and left overnight at 4°C. All the epidermal layers were removed and soaked in 0.25% trypsin for 5 minutes at 37°C, with the reaction stopped by addition of 10% FBS. The tissue was gently pipetted to produce a basal cell suspension. Cells were pelleted by centrifugation and cultured in melanocyte growth medium containing MCDB153 supplemented with 5% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 5 μg/ml insulin, 5 μg/ml transferrin, 0.6 ng/ml bFGF, 10nM endothelin-1, and 10nM α-MSH. The media were changed two or three times weekly until the cells (melanocytes and keratinocytes) were confluent, and then melanocytes were separated from keratinocytes by serial passage (melanocytes react more quickly with trypsin). Detachable melanocytes were removed and cultured on a new tissue culture dish. To expand the population, the melanocytes were cultured in the melanocyte medium, which was changed twice weekly.

To evaluate tyrosinase activity, melanocytes were seeded at $6x10^4$ cells/well of a 96-well plate in melanocyte growth medium and incubated at 37°C in a 5%CO_2 incubator for 3 days. The cells were then cultivated for four days in MCDB153, DMEM, DMEM+kojic acid (500 μg/ml), or DMEM+EGCG (30 μg/ml) with the same supplements. Every 2 days, the medium was replaced with fresh medium and tyrosinase activity was evaluated by L-dopa reaction on day 7.

2.3 Comparison of tyrosinase activity in different media

2.3.1 Quantitative assay of tyrosinase activity

Tyrosinase activity was assayed using the method described by Tomita (Tomita *et al.*, 1992) with some modifications. Briefly, cells were trypsinized, counted, centrifuged, washed with PBS and lysed with 2% triton X in 0.1 M phosphate buffer (PB) at pH 7.2 (400 μl/dish). The lysates were mixed with 200 μl of 0.2% L-dopa (in 0.1M PB) and incubated at 37°C for 1 hour. The absorbance was measured at 405, 450, 475 and 492 nm.

2.3.2 Qualitative evaluation by L-dopa reaction

Tyrosinase activity was determined qualitatively using the L-dopa reaction method (Hirobe *et al.*, 1988), in which L-dopa is oxidized to melanin through catalysis by

tyrosinase. Briefly, cells were fixed with 5% formalin in 0.1 M PB at 4°C for 30 minutes, rinsed with distilled water, and incubated with 0.2% L-dopa solution (in 0.1M PB) at 37°C. After a 3 hours reaction, the solution was replaced with fresh 0.2% L-dopa solution. The cultures were incubated for another hour and then fixed with 10% formalin (in 0.1M PB) at 4°C for 1 hour, after which the color of the cultures was examined visually.

2.4 Statistical analysis

Data are expressed as mean + standard deviation (S.D.) of triplicate samples. Differences between groups were compared using Student *t* test. The *p* value of <0.05 was considered to be statistically significant.

3. Results

3.1 Morphology and growth profile of B16-F10 mouse melanoma cells in different media

Different media containing a variety of ingredients, including amino acids, might affect biological activity. To examine this issue, we first characterized the morphology of B16-F10 cells in DMEM, MEM and RPMI 1640 media, and found no significant difference in morphology for cells cultured in each medium for 7 days in the absence or presence of 300 μg/ml kojic acid. The effect of each medium on cell growth was also investigated, since DMEM contains more glucose (4500 mg/L) than MEM (1000 mg/L) and RPMI 1640 (2000 mg/L). A similar growth pattern of B16-F10 cells was found in all media (Figure 1), with the growth curve showing a lag phase for approximately 3 days and then entering a log phase for 3 days. Cells in each culture medium reached confluence in 7 days.

3.2 Tyrosinase activity of B16-F10 mouse melanoma cells in different media

B16-F10 cells were cultured in DMEM, MEM, and RPMI 1640 in the presence and absence of 300 μg/ml kojic acid for 7 days. Tyrosinase activity was determined quantitatively (from days 4-7) as shown as the optical density per 10^6 cells in Figures 2A and 2B. The cellular tyrosinase activity in the absence of kojic acid increased gradually over time for all media, but the activity in cells cultured in DMEM was significantly higher than in cells cultured in MEM and RPMI 1640 (Figure 2A). Kojic acid at 300 μg/ml (2.11 mM) significantly decreased tyrosinase activity in cells cultured in each medium (Figure 2B) compared to those cultured in the respective medium without kojic acid (Figure 2A).

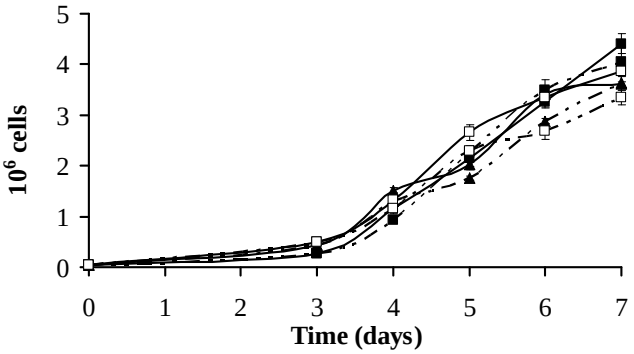

Figure 1. Growth profiles of B16-F10 cells cultured in DMEM (■), MEM (▲) or RPMI1640 (□) in the absence (full line) and presence (broken line) of 300 μg/ml kojic acid for 7 days. Kojic acid was applied on days 3 and 5.

After the cells reached confluence (day 7), the color of the medium was examined for melanin formation. In the absence of kojic acid (control), MEM and RPMI 1640 were much lighter than DMEM, and all media were lighter in the presence of kojic acid (Figure 2C). All cultivated media were darker than control media incubated without cells (data not shown). In cell cultures in which the L-dopa reaction was performed, the presence of kojic acid remarkably decreased the color of the cells (Figure 2D).

3.3 Morphology of human melanocytes in different media

Epidermal cells were isolated from normal human foreskins. The cells were cultured in MCDB153 medium, 5 days later they were attached to the dish surface and started to grow. After 10 days, melanocytes proliferated with dendrites and distributed among keratinocyte colonies (Figure 3A). The cells were subcultured when they were confluent. Melanocytes were rounded up and detached in trypsin solution before keratinocytes, thus with correct timing they could be separated from keratinocytes. Slow proliferation with altered morphology of keratinocytes occurred during cultivation in this medium and after 3 passages, numerous melanocytes with small keratinocyte colonies were seen (Figure 3B). By serial passage, pure melanocytes with less dendrites were obtained (Figure 3C). A portion of melanocytes were cultured in DMEM containing same supplements, 4 days later larger cells with fibroblast-like morphology were seen (Figure 3D).

3.4 Tyrosinase activity of human melanocytes in different media

The L- dopa reaction assay using 96 -well/plates could give noticeably results of tyrosinase activity in melanocytes. To obtain required amount of cells for tyrosinase activity evaluation, melanocytes were cultured in MCDB153 for 2 months. Very low tyrosinase activity was observed in cells cultured in MCDB153 during the culture period as indicated by the light color of the cells (Figure 4A), consistent with the low tyrosine level (3.41 mg/L) in this medium. Interestingly, a significant increase of tyrosinase activity occurred when medium was switched to DMEM, as seen by the dark color of the cells after the L-dopa reaction (Figure 4C). Addition of kojic acid (500 μg/ml = 3.52 mM) had no remarkable

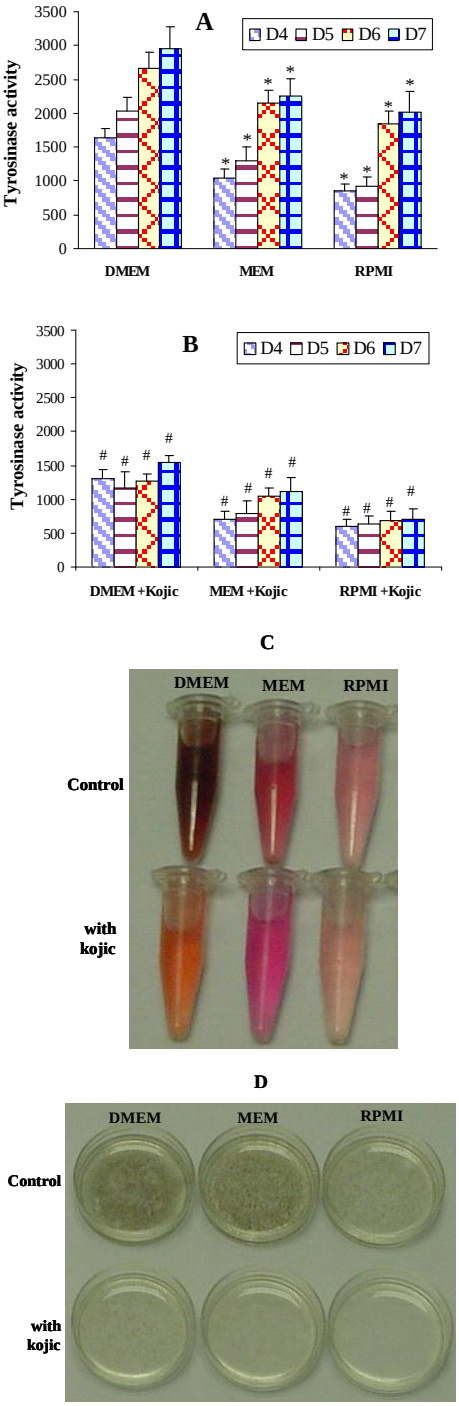

Figure 2. Tyrosinase activity of B16-F10 mouse melanoma cells cultured in DMEM, MEM or RPMI 1640. Tyrosinase activity was determined on days 4-7 by OD measurement at 405 nm and normalized for the number of cells. (A) Activity of cells cultured in each medium on days 4-7 (*, significant difference compared to DMEM, $p < 0.05$). (B) Activity of cells cultured in the presence of 300 μg/ml kojic acid on days 4-7 (#, significant difference compared to the respective medium only, Figure 2A, $p<0.05$). (C) Visual examination of culture media in the absence (control) and presence of kojic acid on day 7. (D) Evaluation of cultured cells on day 7 using a L-dopa reaction.

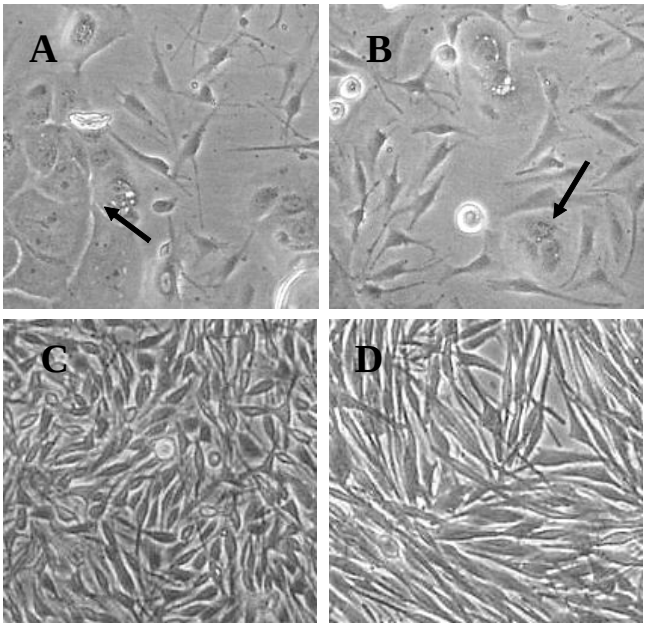

Figure 3. Morphology of epidermal cells isolated from human foreskin and cultured in MCDB153 (A-C) or DMEM. (D) Ten days after initial cultivation in MCDB153 growth medium, melanocytes (small bipolar or tripolar cells) grew and were distributed among the adjacent keratinocyte colonies (arrow). (B) After serial subcultures, the keratinocyte colonies were smaller, whereas the bipolar and tripolar melanocytes were more numerous. (C) Further cultivation in this medium provided a high population density of pure melanocytes. (D) Four days after changing the medium to DMEM with the same supplements, these cells became larger and had a fibroblast-like morphology.

inhibitory effect on tyrosinase activity in cells cultured in MCDB153 (Figure 4B vs. Figure 4A), but decreased tyrosinase activity significantly in cells cultured in DMEM (Figure 4D vs. Figure 4C).

3.5 Effect of EGCG on tyrosinase activity in B16-F10 cells and human melanocytes

In recent years, there has been an intensive research effort on the study of green tea. From this study, the tyrosinase activity of B16-F10 in the presence of EGCG (green tea polyphenol) was evaluated and show in Figure 5. By optical density measurement on day 7, the inhibitory effect of EGCG (5 μg/ml = 10.91 -μM) was 15.86%, and kojic acid (2.11 mM) was 47.88% (Figure 5A). In addition, EGCG slightly decreased the color of the culture medium, whereas kojic acid had a marked effect (Figure 5B). Similar results were obtained for the color of cultured cells after the L-dopa reaction (Figure 5C). From melanocyte study, the results by the L-dopa reaction were in the same direction as B16-F10; kojic acid at 3.52 mM clearly decreased the cell color (Figure 6B), whereas EGCG at 30 μg/ml (65.45 μM) gave a visibly decreased color (Figure 6C) compared to the control (Figure 6A).

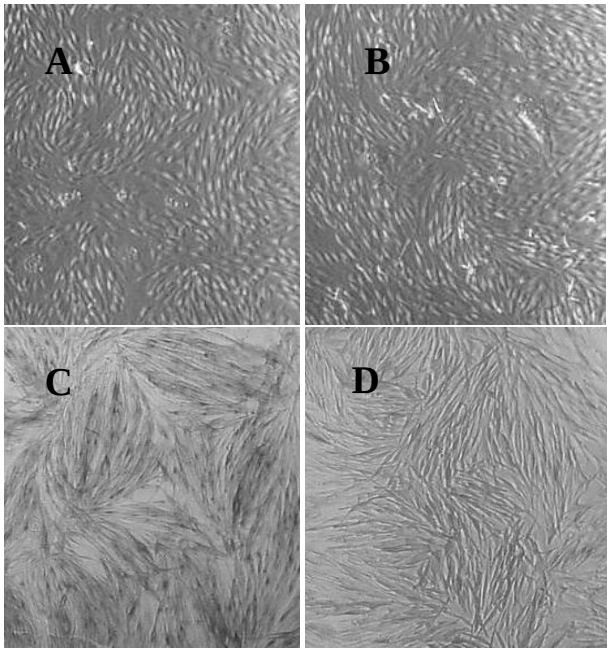

Figure 4. Tyrosinase activity of normal human foreskin melanocytes cultured in different media, evaluated by the L-dopa reaction on day 7. Cells cultured in (A) MCDB153, (B) MCDB153 with 500 µg/ml kojic acid, (C) DMEM, and (D) DMEM with 500 µg/ml kojic acid.

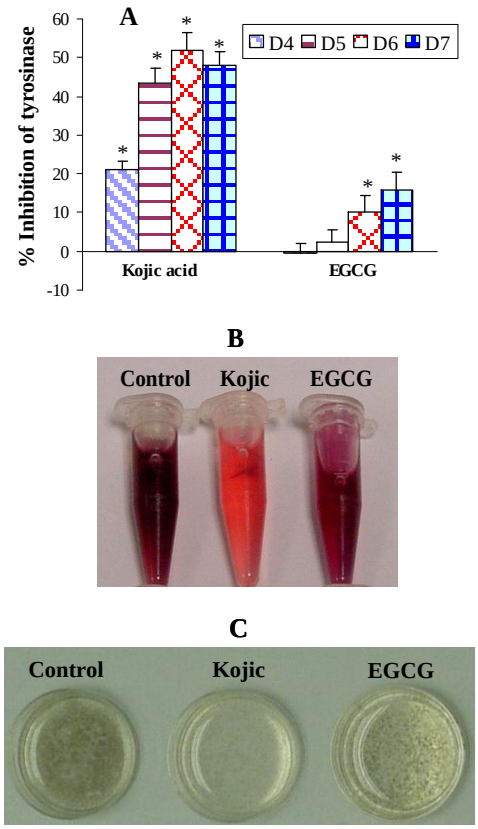

Figure 5. Effect of 5 µg/ml EGCG (compared with 300 µg/ml kojic acid) on tyrosinase activity in B16-F10. (A) Tyrosinase activity was measured on days 4-7 by optical density at 405 nm and normalized for the number of cells. Data are shown as % inhibition compared to medium only (* significant difference compared to medium only, $p<0.05$). (B) Examination of culture media on day 7. (C) Evaluation of cultured cells on day 7 using the L-dopa reaction.

4. Discussion and Conclusion

B16-F10 mouse melanoma cells have been used as a cell model system to study the effects of various compounds on melanogenesis (Kosano *et al.*, 2000; Zhang *et al.*, 1992; Jacobsohn *et al.*, 1998). This cell system has been grown in various culture media; however, their effect on the melanogenic activity of cells has not been investigated. We therefore tested the effect of various commonly used culture media, including DMEM, MEM, and RPMI 1640, on melanoma cell growth and morphology. The results showed that these media with or without kojic acid (300 µg/ml) had no appreciable effects on the morphology and growth pattern of B16-F10 mouse melanoma cells.

Tyrosinase activity is commonly used as a key indicator of melanogenesis, which involves the conversion of tyrosine to L-dopa by tyrosinase, with subsequent oxidation to melanin. In the present study, we examined the effect of culture media on cellular tyrosinase activity by measuring the optical density and visual examination of the melanin color product produced from the catalysis of L-dopa. One issue that we encountered in performing the optical density measurements is the determination of optimal wavelength for melanin absorption studies. Previously, several wavelengths ranging from 405 nm to 492 nm have been used for this assay (Nagajima *et al.*, 1998; Wang *et al.*, 2006; Kosano *et al.*, 2000; Hedley *et al.*, 1998). In the present study, we found that the wavelength of 405 nm gave the highest optical density (data not shown). Using this wavelength, we further

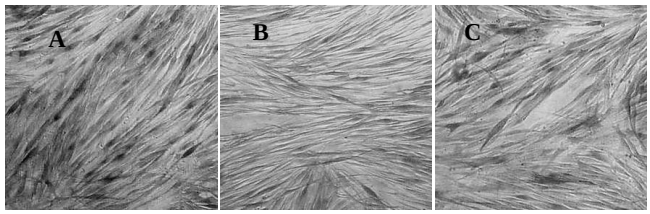

Figure 6. Effect of 30 µg/ml EGCG (compared with 500 µg/ml kojic acid) on tyrosinase activity in normal human foreskin melanocytes, with activity evaluated by the L-dopa reaction on day 7. Cells cultured in (A) DMEM, (B) DMEM with kojic acid, and (C) DMEM with EGCG.

showed that the optical density or tyrosinase activity of B16-F10 cells was greatest in DMEM, followed by MEM and RPMI 1640, respectively. These results were supported by visual inspection of the color products in cells and culture media. The likely reason that DMEM gave the strongest tyrosinase activity is the tyrosine content in the medium.

Table 1. Components in different media used in the study.

Media	DMEM	MEM	RPMI 1640	MCDB153
Component	mg/L	mg/L	mg/L	mg/L
D-Glucose	4500.00	1000.00	2000.00	1081.000
L-Glutamine	584.00	292.00	300.00	877.200
Glycine	30.00	7.50	10.00	7.510
L-Isoleucine	105.00	52.00	50.00	1.968
L-Leucine	105.00	52.00	50.00	65.600
L-Lysine · HCL	146.00	72.50	40.00	18.270
L-Methionine	30.00	15.00	15.00	4.480
L-Phenylalanine	66.00	32.00	15.00	4.960
L-Serine	42.00	10.50	30.00	63.060
L-Threonine	95.00	48.00	20.00	11.910
L-Tyrosine · 2Na · $2H_2O$	104.00	52.00	29.00	3.410

DMEM contains 104 mg/L of tyrosine, whereas MEM and RPMI 1640 contain 52 and 29 mg/L, respectively. These results indicate that tyrosinase activity may be upregulated by the presence of tyrosine, the finding that is consistent with a previous report showing that increasing tyrosine level in the medium results in elevated tyrosinase activity (Slominski *et al.*, 1988).

Further studies were performed using normal human melanocytes, which better mimic the *in vivo* conditions than the mouse melanoma cells. However, in expanding cell population, melanocytes require low tyrosine-containing media such as MCDB153 in order to avoid cell differentiation. Having shown that B16-F10 cultured in DMEM gave the highest tyrosinase activity, human melanocytes were also cultured in DMEM and their morphology was examined and compared with those grown in MCDB153. It was found that slow proliferation with altered morphology occurred when melanocytes were cultured in DMEM, a finding that is consistent with a previous report (Shawahn *et al.*, 2001) showing that a low tyrosine medium (25-30 μM) stimulated melanocyte proliferation while a high tyrosine medium (3.7 mM) accelerated differentiation. As melanocytes exhibited an altered morphology in DMEM, which might affect biological activities, this medium is not recommended for expansion of melanocyte cell population in culture.

Due to the lack of neonatal foreskins obtained from routine circumcision and the limited proliferative capacity of primary human melanocytes**,** the tyrosinase activity of these cells was studied via the L-dopa reaction only because this method requires minimal cell number for analysis (i.e., $1x10^5$ cells/sample**)** while the suitable number for optical density analysis is about $5x10^6$ cells/sample to obtain reliable results. Even though cultured melanocytes showed a very low tyrosinase activity in MCDB153, such activity was greatly increased when the culture medium was changed to DMEM. To evaluate the efficacy of test substances on melanocytes, a positive control with known tyrosinase-modulating activity is generally needed. In this study, we used kojic acid, which is a known inhibitor of tyrosinase, to aid the study of cell culture medium effect on tyrosinase activity. The results showed that kojic acid significantly inhibited the enzyme activity in both B16-F10 and melanocytes in DMEM. These results suggest that DMEM is a suitable medium for sensitive evaluation of the tyrosinase activity in B16-F10 and melanocytes.

Plant-based de-pigmenting agents with reduced toxicity are of current interest, and among these the green tea polyphenol, EGCG, has been reported to inhibit mushroom tyrosinase activity with an IC_{50} of 34.1 μM (No *et al.*, 1999). However, *in vitro* cellular studies of the effects of EGCG on pigmentation have not been reported. Therefore, we investigated the tyrosinase inhibitory effects of EGCG in B16-F10 cells and human melanocytes cultured in DMEM, and the results were compared with the reported inhibitory effect of EGCG on mushroom tyrosinase activity, using maximum non-cytotoxic concentration (Diawpanich *et al.*, unpublished). The results show that EGCG could inhibit tyrosinase activity in both B16-F10 and human melanocytes. The potency of inhibition in these cells was comparable to that reported in mushroom tyrosinase (No *et al.*, 1999), i.e., a 3-times lower concentration of EGCG (10.91 μM *vs*. 34.1 μM) had about a third of the inhibitory effect (15.86% *vs*. 50%) observed in mushroom tyrosinase

In summary, we have developed an *in vitro* cell model system for melanogenesis studies using normal human foreskin melanocytes and mouse B16-F10 melanoma cells. DMEM was found to be the optimal medium for tyrosinase activity assay in these cells since it allows sensitive detection of the enzyme activity, which is crucial for assessment of the inhibitory effects of phytochemicals such as EGCG.

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