Stimulation of Melanogenesis by Nordihydroguaiaretic Acid in Human Melanoma Cells

Susumu Takekoshi¹, Hidetaka Nagata¹ and Kanae Kitatani¹

¹Department of Cell Biology, Division of Host Defense Mechanism, Tokai University School of Medicine, 143 Shimokasuya, Isehara, Kanagawa 259–1193, Japan

Received May 28, 2014; accepted July 8, 2014; published online September 2, 2014

Nordihydroguaiaretic acid (NDGA), a lignan found in vegetables, fruits and legumin, has been shown to possess antineoplastic, antiviral and antioxidant characteristics. In this study, we examined the effect of NDGA on melanogenesis in human melanoma cells (HMVII). In vitro, NDGA does not alter mushroom tyrosinase activity. However, in NDGA-treated HMVII cells, cellular tyrosinase activity increased in both a time- and dose-dependent manner. The concomitant increases in melanin content in NDGA-treated cells indicated an elevation of melanin synthesis by tyrosinase activation. In addition, after a 7-day incubation, melanin content in 20 μM NDGA-treated cells increased 5.02 fold. Tyrosinase protein also increased by treatment with NDGA. Nevertheless, tyrosinase mRNA was not altered in NDGA-treated cells. Our results suggest that NDGA can increase tyrosinase activity and de novo synthesis of melanin in human melanoma cells. We found that NDGA is a novel potent stimulator of melanogenesis in human melanoma cells.

Key words: nordihydroguaiaretic acid, tyrosinase, melanin, melanogenesis, HMVII melanoma cells

I. Introduction

A phenolic lignan, nordihydroguaiaretic acid (NDGA), long known as a natural product, is distributed widely in the plant kingdom. NDGA was originally extracted from Larrea divaricata shrubs and used as an antioxidant in processed meat, dairy products and baked goods [33]. NDGA exhibits a broad range of biological properties including anti-tumor, anti-viral and anti-oxidant properties [10, 13, 22].

NDGA has a polyphenolic structure, as do flavonoids, such as quercetin and epigallocatechin. Quercetin and epigallocatechin possess the regulatory effects of mushroom tyrosinase in vitro [4, 31]. Furthermore, we demonstrated that quercetin has a melanogenic effect on a human melanoma cell line of vagina II (HMV-II), a three-dimensional human epidermal model, and hair follicles of the mouse [28, 37, 38]. However, no study on melanogenesis regulation by NDGA at the cellular level has been reported. To regulate melanogenesis, cells are equipped with melanogenic enzymes such as tyrosinase, tyrosine-related protein 1 (TRP1) and tyrosine-related protein-2 (TRP-2). Tyrosinase, the first enzyme described to regulate melanogenesis, initiates melanogenesis by catalyzing the conversion of tyrosine to L-3,4-dihydroxyphenylalanine (DOPA) and dopaquinone. Melanin synthesis is triggered by melanogens, a large group of melanocyte-stimulating factors, such as endothelin-1, α-melanocyte-stimulating hormone (α-MSH), isobutyl-methyl-xanthine (IBMX), ultraviolet light B (UVB) irradiation, 5-methoxypsoralen with UV (PUVA) and histamine [1, 7, 9, 15, 25, 26]. Whether the regulation of those melanogenic enzymes occurs by modulation of synthetic rate of proteins or mRNA levels is not clearly established.

In the present report, we demonstrated that NDGA enhances melanogenesis in HMVII melanoma cells. Our results indicate that treatments with NDGA led to increased...
tyrosinase activity and de novo synthesis of melanin in HMVII cells. To fully interpret these results, we discuss the regulation of tyrosinase at the levels of expression and enzyme activity.

II. Materials and Methods

Chemicals

Nordihydroguaiaretic acid, mushroom tyrosinase, melanin and L-dopa were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Enzymatic assay of tyrosinase

Tyrosinase activity using L-dopa as the substrate was assayed spectrophotometrically. A 25 μl sample of NDGA was added to the assay mixture containing 5 mM L-dopa solution, 50 mM phosphate buffer (pH 7.4) and 25 μl of 0.6 mg/ml mushroom tyrosinase solution which was added to a 96-well microplate for a total volume of 200 μl. The assay mixture was incubated at 37°C for 10 min. After incubation, the absorbance was measured at 475 nm in a model SPECTRAmax 250 microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Melanoma culture

Melanocytes are specialized cells in the skin that produce melanin, a pigment that is responsible for skin and hair color and that provides protection against ultraviolet (UV) radiation. Melanoma is a malignant tumor arising from the melanocyte lineage [14, 24, 40, 41]. HMVII is a human melanoma cell line that was established from a black-brown malignant melanoma in the vaginal wall [11, 29]. HMVII cells were kindly provided by the RIKEN Cell Bank. HMVII cells were cultured in Ham’s F12 medium containing 10% heat-inactivated fetal bovine serum (FBS) in a humidified 37°C atmosphere consisting of 5% CO₂ and 95% air. Cell plating densities were arranged so that those cells were in log phase of growth for the duration of incubation with drug. Subcultures of cells were plated at a density of 4×10⁴ cells/cm². Approximately 24 hr later, fresh medium and quercetin were added, and cells were harvested 7 days after drug addition. Tested quercetin was dissolved in dimethylsulfoxide (DMSO). The final DMSO concentration was 0.1% in all experiments (including control) and it had no measurable effect on HMVII cells.

Melanin assay

After washes in phosphate-buffered saline (PBS), cells were detached by short incubation in trypsin/EDTA (0.05%/0.02% in PBS). An aliquot was used for the cell count. The remaining cells were sonicated and incubated overnight in 500 μl 1 M NaOH. Melanin concentration was calculated by comparison of the OD at 475 nm of unknown samples with a standard curve obtained with synthetic melanin.

Assay of cellular tyrosinase activity

Cellular tyrosinase activity using L-dopa as the substrate was assayed by the method of Maeda et al. [23]. Cells were washed with PBS and lysed with 45 μl of 1% Triton X-100-PBS. After sonication, 5 μl of 20 mM L-dopa was added to the wells. The plates were incubated at 37°C for 60 min, and the absorbance was measured at 475 nm in a microplate reader. The absorbance values were compared with a standard curve obtained with mushroom tyrosinase; the standard curve was linear within the range of experimental values.

L-DOPA staining of electrophoresed gels

To identify the amount of L-DOPA-positive tyrosinase and the effect of glycosylation of tyrosinase, L-DOPA staining of the electrophoresed gel was performed as follows. After incubation with quercetin, treated cells were solubilized in 0.1 M sodium phosphate buffer (PB) (pH 6.8) containing 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 μg/ml aprotinin, and 10 μg/ml leupeptin without mercaptoethanol and without heating. Protein content was measured using the Bio-Rad protein assay kit (Bio-Rad. Richmond, CA, USA) with bovine serum albumin (BSA) as a standard. Gels containing tyrosinase activity were placed in a flat-bottom container with 200 ml of rinse buffer (0.1 M PB, pH 6.8) and equilibrated for 30 min at room temperature by gently shaking. The rinse buffer was then drained from the gels and the gels were transferred into 200 ml of staining solution, which contained the rinse buffer supplemented with 5 mM L-DOPA, and incubated in the dark for 30 min at 37°C. Tyrosinase activity was visualized in the gels as dark bands containing DOPA-melanin [21].

Immunoblot analysis

Cell lysates were solubilized in 2% SDS containing 10% 2-mercaptoethanol, electrophoresed in SDS-10% polyacrylamide gels and then electro-transferred to nitrocellulose membranes [20, 39]. The membranes were blocked with 5% non-fat dry milk and 0.1% Tween 20 in 0.01 M tris-buffered saline (TBS) for 1 hr at room temperature. After 3 washes with TBS containing 0.1% Tween 20 (T-TBS), membranes were incubated with anti-MAT-1 antibody, which recognizes the COOH terminal of tyrosinase (POLA R&D Lab, Yokohama, Japan), 1:250, diluted with TBS containing 5% BSA and 0.1% Tween 20 for 1 hr at room temperature. After 5 washes with T-TBS, the membranes were incubated with horseradish peroxidase-conjugated anti-mouse Ig antibody (Amersham Co. Tokyo, Japan), 1:5000 dilution with T-TBS, for 1 hr at room temperature. After 5 washes with T-TBS, bands were detected by enhanced chemiluminescence method (Amersham Co. Tokyo, Japan).

RNA preparation and northern blot analysis

Total RNA was isolated from cells by the guanidine
isothiocyanate-CsCl method [6]. Ten micrograms of total RNA was electrophoresed on 1% agarose gels containing 18% formaldehyde and blotted onto nitrocellulose filters in 10×SSC (1× = 0.15 M NaCl, 0.015 M sodium citrate). cDNA probe for human tyrosinase (pRHOHT2) was generously provided by the RIKEN Gene Bank. Tyrosinase cDNA [36] was labeled with $^{32}$P with a random priming kit (TAKARA BIO INC, Shiga Japan). The cDNA probe for chicken β-actin was used as an internal positive control (housekeeping gene mRNA). The filters were prehybridized at 42°C for 4 hr in a mixture comprising 50% formamide, 0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 5×SSC, 0.1% sodium lauroylsarcosine, 0.05 M sodium phosphate (pH 6.5) and 0.1 mg/ml denatured salmon sperm DNA. Hybridization was performed at 42°C for 18 hr with heat-denatured $^{32}$P-labeled DNA probes in the same solution as described above. Thereafter, they were washed with 2×SSC containing 0.1% SDS at room temperature for 3 times. The total radioactivity of each hybridized band was quantified using a Fujix bio-Image analyzer BAS2000 (Fuji Photo Film Co. Ltd., Tokyo, Japan).

Immunocytochemistry

HMV-II cells were plated on coverslips and cultured for 7 or 14 days in medium containing Lignan. Cells were fixed with 4% PFA-PBS for 10 min, permeabilized with 0.05% Triton X-100 in PBS for 5 min and blocked with 5 mg/ml BSA in PBS for 30 min at room temperature. Cells were incubated with tyrosinase antibody, 1:500, diluted with 1 mg/ml BSA-PBS at 4°C overnight. After 3 washes, the signal was amplified with biotin-conjugated anti-mouse Ig antibody (Fitzgerald Industries, North Acton, MA, USA) and Vectastain ABC-AP kit (Vector Labs, Burlingame, CA, USA). Immunoreactivity was visualized by Fast Red RR Salt (Sigma Aldrich, St Louis, MO, USA) and naphthol AS-BI phosphate disodium salt (Sigma Aldrich) in 0.05 M propanediol buffer (pH 9.8).

III. Results

Effect of NDGA on mushroom tyrosinase activity

We have tested the effect of NDGA on the activity of mushroom tyrosinase using L-DOPA as the substrate. As shown in Figure 1, NDGA has no direct effect on the activity of mushroom tyrosinase.

Effect of NDGA on melanogenesis in HMVII cells

We examined the effect of NDGA on melanogenesis in HMVII melanoma cells. On treating HMVII cells with 20 μM NDGA for 7 days, there was a striking increase in the average degree of melanization, seen as pigmentation, in the NDGA-treated cultures (Fig. 2).

The results of quantitative measurements of melanin content are shown in Figure 3. These results correlate with the visible appearance shown in Figure 2. In NDGA-treated
NDGA increased the melanin content in HMVII. (a) Melanin content was determined by measuring the absorbance at 475 nm of HMVII cells treated with various concentrations of NDGA for 7 days as described in Materials and Methods. (b) Cells were treated with 20 μM NDGA for 1, 3, 5 and 7 days. Each value of melanin content is the mean±SD of 5 determinations. Significant differences were determined by Student’s t-test; *P<0.01, **P<0.001.

Fig. 3.

NDGA enhanced tyrosinase activity in HMVII. (a) Tyrosinase activity was measured using L-DOPA (1 mM) as the substrate. Cells were treated with 20 μM NDGA for different time periods. (b) Cells were treated with various concentrations of NDGA for 7 days. Tyrosinase activity is the mean±SD of 5 determinations. Significant differences were determined by Student’s t-test; *P<0.01, **P<0.0001.

Fig. 4.

Effect of NDGA on cellular tyrosinase activity in HMVII cells

To investigate the effects of NDGA on tyrosinase activity, HMVII cells were incubated with various concentrations of NDGA for 7 days prior to harvesting and tyrosinase activity was assayed. Tyrosinase activity of HMVII cells increased in a dose-dependent manner by the addition of NDGA to the culture medium (Fig. 4a). In NDGA-treated cells, tyrosinase activity significantly increased 4.05 fold at 1 μM (P<0.01), 7.24 fold at 5 μM (P<0.001), 14.48 fold at 10 μM (P<0.001) and 36.74 fold at 20 μM (P<0.0001) of drug concentration as compared to DMSO-treated control cells (Fig. 4a). In addition, after several days (3, 5 and 7 days) incubation, NDGA-treated cells also markedly increased 14.2, 27.9, 31.1 and 36.7 fold, respectively (Fig. 3b).

Expression of tyrosinase was increased by NDGA treatment

Immunoblotting of tyrosinase revealed a single band of protein with a molecular weight approximately 70 kD. Immunoblotting analyses indicated that the tyrosinase protein level was increased by NDGA, which correlated well with DOPA reactivity (Fig. 5c, d).

Effect of NDGA on tyrosinase mRNA expression in HMVII cells

To confirm the immunoblot results, we analyzed tyrosinase mRNA from HMVII cells treated with NDGA (Fig. 6). NDGA had little or no effect on tyrosinase mRNA levels after 1- to 5-day incubations. After 7 days, tyrosinase mRNA was hardly detectable compared to the basal and 1- to 5-day NDGA-treated levels. Also, β-actin mRNA was not affected by NDGA in HMVII cells.
Immunohistochemical study

Immunohistochemistry of tyrosinase was performed. Staining intensity of tyrosinase protein was clearly increased by NDGA treatment (Fig. 7).

IV. Discussion

In this study, we demonstrated that NDGA increased intracellular melanin content and tyrosinase activity in HMVII melanoma cells. Our results appear unique in that actions of NDGA were manifested in the absence of UV irradiation. In many cases, the actions of 5-methoxypsoralen and 8-methoxypsoralen were active in stimulating melanogenesis in the presence of UV irradiation [19, 26].

When HMVII melanoma cells were submitted to NDGA over several days, tyrosinase activity and melanin content were stimulated in time- and dose-dependent manners. As compared with control, melanin content was increased about 5.02 fold by treatment with 20 μM NDGA for 7 days. Tyrosinase activity was also increased, to 36.74-fold higher than control. These results may demonstrate that the ratio of melanin formation (M) to tyrosinase activity (T) is low. This is consistent with melanogenesis induced by high concentrations of 5-methoxypsoralen (5-MOP) [25]. A high M/T ratio would suggest a post-tyrosinase activation of melanogenesis, whereas a low ratio should reflect the presence of blocking activity [12, 18]. Our results for the M/T ratio suggested that this blocking activity might be suppressed by NDGA with a consequent enhancement of melanogenesis.

There have been reports on the relationship between tyrosinase activity and the tyrosinase mRNA level using α-MSH, PUVA and glucocorticoid [9, 25]. In contrast, the correlation between tyrosinase mRNA and tyrosinase activity has not been demonstrated using PUVA, UV and fatty acid [3, 26, 27], suggesting that post-transcriptional regulation of tyrosinase and/or other events may play an essential role in melanogenesis.

Fig. 5. DOPA staining of tyrosinase (a, b) and Western blot analysis of tyrosinase (c, d) from HMVII after treatment with NDGA. Cells were treated with NDGA (1, 5, 10 and 20 μM) for different time periods (1, 3, 5 and 7 days). (a, b) Cell extracts were electrophoresed on SDS-10% polyacrylamide gel and incubated with 5 mM L-DOPA at 37°C. (c, d) Protein extracts were immuno-blotted with anti-tyrosinase antibody (MAT-1) as described in Materials and Methods. (a, c) Lane 1, nontreated cells; lane 2, DMSO-treated cells; and Lanes 3, 4, 5, and 6, NDGA 1, 5, 10 and 20 μM for 7 days, respectively. (b, d) Lane 1, nontreated cells; lane 2, DMSO-treated cells; Lanes 3, 4, 5, and 6, NDGA 20 μM, 1, 3, 5 and 7 days, respectively.

Fig. 6. Expression of tyrosinase mRNA after NDGA treatment of HMVII. Cells were treated with NDGA for 1, 3, 5 and 7 days. Total RNA (10 μg) was fractionated on formalin-agarose gels, transferred to nitrocellulose membranes and then hybridized with 32P-labelled cDNA for human tyrosinase (pRHOHT2) before subsequent hybridization of the same blot with chicken β-actin as a control, as described in Materials and Methods. (a) Lane 1, nontreated cells; lane 2, DMSO-treated cells; lanes 3, 4, 5, and 6, NDGA 1, 5, 10 and 20 μM for 7 days, respectively. (b) Lane 1, nontreated cells; lane 2, DMSO-treated cells; and lanes 3, 4, 5, 6, NDGA 20 μM, 1, 3, 5 and 7 days, respectively.
role in the rate of melanin synthesis. Whether these contradictory results were due to the participation of a pre-existing pool of inactive tyrosinase [17] or due to the removal of tyrosinase inhibitor [8] has not been determined yet. An explanation for this apparent discrepancy may be the stability of tyrosinase mRNA. Also, an increase in tyrosinase mRNA may occur early after NDGA treatment, but because of rapid turnover of the transcript may no longer have been detectable 24 hr later when our analysis was performed. To unravel these hypotheses, further studies such as determination of mRNA stability are required.

We found that cellular tyrosinase activity was increased after NDGA treatment of HMVII cells while the mushroom tyrosinase activity was unaffected by NDGA. Our results show specifically that NDGA, like UV A irradiation [7] or other melanogenic inducers such as MSH or IBMX [9, 17], involved, at least in part, an increase in de novo synthesis of tyrosinase to enhance melanogenesis in HMVII cells.

It is well known that NDGA possesses scavenging (or quenching) abilities against free radicals and active oxygen species. Postaire et al. reported that antioxidant nutrients such as vitamin E and carotene induced pigmentation in mouse skin [35]. In addition, Jiménez-Cervantes et al. reported that H$_2$O$_2$ inhibited melanogenesis in melanoma cells [16]. Furthermore, in several pathological conditions, like vitiligo, the generation of free radicals and/or the resulting increase in lipid peroxidation products have been proposed as etiological factors for depigmentation [34]. Nakayama et al. reported that NDGA has suppressive effects against cytotoxicity caused by H$_2$O$_2$ [30]. H$_2$O$_2$ suppression by NDGA may play an important role in the modulation of melanogenesis.

Nylander et al. reported that tyrosinase and tyrosinase-related protein-1 are activated by wild type p53 linked UV irradiation [32]. NDGA was reported to stabilize wild type p53 protein [2]. NDGA-evoked melanogenesis may be due to the accumulation of wild type p53 protein. The role of wild type p53 in the stimulating effect of NDGA on melanogenesis has provoked much interest.

Melanogenesis can affect chemotherapy and radiotherapy for melanoma. Anna et al. has reported that the melanogenesis affects the clinical outcome in melanoma patient [5]. They have demonstrated that melanogenesis shorten overall survival and disease-free survival times in patient with metastatic melanoma. Since NDGA markedly
stimulate melanogenesis in melanoma cell line, judicious use of NDGA for the therapy of human skin disease would be advocated.

In the present study, we demonstrated that NDGA could be a potent natural melanogenic inducer. Our results show that NDGA involves, at least in part, increased tyrosinase activity to enhance melanogenesis in human melanoma cells. Although the biologic impact of NDGA in human melanogenesis is still a matter of debate, NGDA may be a potential chemical to treat dermatological diseases such as vitiligo and to ameliorate poliosis. The importance of NDGA as a new medicine will be elucidated in a future study.

V. Abbreviations

DMSO, dimethylsulfoxide; DOPA, L-3,4-dihydroxyphenylalanine; PUVA, 5-methoxypsoralen plus UVA; MSH, melanocyte-stimulating hormone NDGA, nordihydroguaiaretic acid

VI. Acknowledgments

The authors thank Takashi Kasuga for providing HMVII cell lines; Shigeki Shibahara for tyrosinase cDNA probe pRHOHT2; and Johbu Itoh and Hiroshi Kamiguchi for invaluable technical advice. This work was supported by a Grant-in-Aid for Scientific Research (C) (no. 20590385) from the Japanese Society for the Promotion of Science.

VII. References

1. Aberdam, E., Romero, C. and Ortonne, J. P. (1993) Repeated UVB irradiations do not have the same potential to promote stimulation of melanogenesis in cultured normal human melanocytes. J. Cell. Sci. 106; 1015–1022.
2. Allen, K. L., Tschantz, D. R., Awad, K. S., Lynch, W. P. and DeLucia, A. L. (2007) A plant lignan, 3'-O-methyl-nordihydroguaiaretic acid, suppresses papillomavirus E6 protein function, stabilizes p53 protein, and induces apoptosis in cervical tumor cells. Mol. Carcinog. 46; 564–575.
3. Ando, H., Itoh, A., Mishima, Y. and Ichihashi, M. (1995) Correlation between the number of melanosomes, tyrosinase mRNA levels, and tyrosinase activity in cultured murine melanoma cells in response to various melanogenesis regulatory agents. J. Cell. Physiol. 163; 608–614.
4. Arung, E. T., Wijaya Kusuma, I., Shimizu, K. and Kondo, R. (2011) Tyrosinase inhibitory effect of quercetin 4’-O-beta-D-glucopyranoside from dried skin of red onion (Allium cepa). Nat. Prod. Res. 25; 256–263.
5. Brozyna, A. A., Jozwicki, W., Carlson, J. A. and Slominski, A. T. (2013) Melanogenesis affects overall and disease-free survival in patients with stage III and IV melanoma. Hum. Pathol. 44; 2071–2074.
6. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. and Rutter, W. J. (1979) Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18; 5294–5299.
7. Friedmann, P. S. and Gilchrest, B. A. (1987) Ultraviolet radiation directly induces pigment production by cultured human melanocytes. J. Cell. Physiol. 133; 88–94.
8. Halaban, R., Pomerantz, S. H., Marshall, S., Lambert, D. T. and Lerner, A. B. (1983) Regulation of tyrosinase in human melanocytes grown in culture. J. Cell Biol. 97; 480–488.
9. Halaban, R., Pomerantz, S. H., Marshall, S. and Lerner, A. B. (1984) Tyrosinase activity and abundance in Cloudman melanoma cells. Arch. Biochem. Biophys. 230; 383–387.
10. Harper, A., Kerr, D. J., Gescher, A. and Chipman, J. K. (1999) Antioxidant effects of isoflavonoids and lignans, and protection against DNA oxidation. Free Radiat. Res. 31; 149–160.
11. Hasumi, K., Sakamoto, G., Sugano, H., Kasuga, T. and Masubuchi, K. (1978) Primary malignant melanoma of the vagina: study of four autopsy cases with ultrastructural findings. Cancer 42; 2675–2686.
12. Hearing, V. J., Korner, A. M. and Pawelek, J. M. (1982) New regulators of melanogenesis are associated with purified tyrosinase isozymes. J. Invest. Dermatol. 79; 16–18.
13. Hwu, J. R., Tseng, W. N., Gnaabre, J., Giza, P. and Huang, R. C. (1998) Antiviral activities of methylated nordihydroguaiaretic acids. 1. Synthesis, structure identification, and inhibition of tat-regulated HIV transactivation. J. Med. Chem. 41; 2994–3000.
14. Ikeda, R., Murakoshi, M., Ohtani, M., Tagawa, M., Mikami, T., Natsume, T., Watanabe, J., Kobayashi, M. and Nakayama, T. (2000) Induction of apoptosis in mice with B16 melanoma by treatment with the antimicrotubule agent TCTT-1027. Acta Histochem. Cytochem. 33; 341–347.
15. Imokawa, G., Miyagishi, M. and Yada, Y. (1995) Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UBV-exposed human epidermis. J. Invest. Dermatol. 105; 32–37.
16. Jimenez-Cervantes, C., Martinez-Esparza, M., Perez, C., Daum, N., Solano, F. and Garcia-Borron, J. C. (2001) Inhibition of melanogenesis in response to oxidative stress: transient downregulation of melanocyte differentiation markers and possible involvement of microphthalmia transcription factor. J. Cell Sci. 114; 2335–2344.
17. Jimenez, M., Kameyama, K., Maloy, W. L., Tomita, Y. and Hearing, V. J. (1988) Mammalian tyrosinase: biosynthesis, processing, and modulation by melanocyte-stimulating hormone. Proc. Natl. Acad. Sci. U S A 85; 3830–3834.
18. Kameyama, K., Takemura, T., Hamada, Y., Sakai, C., Kondoh, S., Nishiyama, S., Urabe, K. and Hearing, V. J. (1993) Pigment production in murine melanoma cells is regulated by tyrosinase, tyrosinase-related protein 1 (TRP1), DOPAchrome tautomerase (TRP2), and a melanogenic inhibitor. J. Invest. Dermatol. 100; 126–131.
19. Kao, C. H. and Yu, H. S. (1992) Comparison of the effect of 8-methoxypsoralen (8-MOP) plus UVA (PUVA) on human melanocytes in vitiligo vulgaris and in vitro. J. Invest. Dermatol. 98; 734–740.
20. Laemmlli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227; 680–685.
21. Laskin, J. D. and Piccinini, L. A. (1986) Tyrosinase isozyme heterogeneity in differentiating B16/C3 melanoma. J. Biol. Chem. 261; 16626–16635.
22. Lu, J. M., Nurko, J., Weakley, S. M., Jiang, J., Kougiis, P., Lin, P. H., Yao, Q. and Chen, C. (2010) Molecular mechanisms and clinical applications of nordihydroguaiaretic acid (NDGA) and its derivatives: an update. Med. Sci. Monit. 16; RA93–100.
23. Maeda, K. and Fukuda, M. (1996) Arbutin: mechanism of its depigmenting action in human melanocyte culture. J. Pharmacol. Exp. Ther. 276; 765–769.
24. Matsuta, M., Matsuta, M., Akasaka, T. and Kagabu, T. (1999) Changes in the cyclin D1 gene copy number in melanoma cell lines detected by double-target fluorescence in situ hybridization.
Acta Histochem. Cytochem. 32; 431–436.

25. Mengeaud, V. and Ortonne, J. P. (1994) Regulation of melanogenesis induced by 5-methoxypsoralen without ultraviolet light in murine melanoma cells. Pigment Cell Res. 7; 245–254.

26. Mengeaud, V. and Ortonne, J. P. (1996) PUVA (5-methoxypsoralen plus UVA) enhances melanogenesis and modulates expression of melanogenic proteins in cultured melanocytes. J. Invest. Dermatol. 107; 57–62.

27. Naeyaert, J. M., Eller, M., Gordon, P. R., Park, H. Y. and Gilchrest, B. A. (1991) Pigment content of cultured human melanocytes does not correlate with tyrosinase message level. Br. J. Dermatol. 125; 297–303.

28. Nagata, H., Takekoshi, S., Takeyama, R., Homma, T. and Osamura, R. Y. (2004) Quercetin enhances melanogenesis by increasing the activity and synthesis of tyrosinase in human melanoma cells and in normal human melanocytes. Pigment Cell Res. 17; 66–73.

29. Nakamura, T., Seki, S., Matsubara, O., Ito, S. and Kasuga, T. (1988) Specific incorporation of 4-S-cysteinylphenol into human melanoma cells. J. Invest. Dermatol. 90; 725–728.

30. Nakayama, T., Hori, K., Osawa, T. and Kawakishi, S. (1992) Suppression of hydrogen peroxide-induced mammalian cytotoxicity by nordihydroguaiaretic acid. Biosci. Biotechnol. Biochem. 56; 1162–1163.

31. No, J. K., Soung, D. Y., Kim, Y. J., Shim, K. H., Jun, Y. S., Rhee, S. H., Yokozawa, T. and Chung, H. Y. (1999) Inhibition of tyrosinase by green tea components. Life Sci. 65; PL241–246.

32. Nylander, K., Bourdon, J. C., Bray, S. E., Gibbs, N. K., Kay, R., Hart, I. and Hall, P. A. (2000) Transcriptional activation of tyrosinase and TRP-1 by p53 links UV irradiation to the protective tanning response. J. Pathol. 190; 39–46.

33. Olivetto, E. (1972) Nordihydroguaiaretic acid, A naturally occurring antioxidant. Chem. Ind. 2; 677–679.

34. Passi, S., Grandinetti, M., Maggio, F., Stancato, A. and De Luca, C. (1998) Epidermal oxidative stress in vitiligo. Pigment Cell Res. 11; 81–85.

35. Postaire, E., Jungmann, H., Bejot, M., Heinrich, U. and Tronnier, H. (1997) Evidence for antioxidant nutrients-induced pigmentation in skin: results of a clinical trial. Biochem. Mol. Biol. Int. 42; 1023–1033.

36. Takeda, A., Tomita, Y., Okinaga, S., Tagami, H. and Shibahara, S. (1989) Functional analysis of the cDNA encoding human tyrosinase precursor. Biochem. Biophys. Res. Commun. 162; 984–990.

37. Takekoshi, S., Matsuzaki, K. and Kitatani, K. (2013) Quercetin stimulates melanogenesis in hair follicle melanocyte of the mouse. Tokai J. Exp. Clin. Med. 38; 129–134.

38. Takeyama, R., Takekoshi, S., Nagata, H., Osamura, R. Y. and Kawana, S. (2004) Quercetin-induced melanogenesis in a reconstituted three-dimensional human epidermal model. J. Mol. Histol. 35; 157–165.

39. Towbin, H., Staehelin, T. and Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl. Acad. Sci. U S A 76; 4350–4354.

40. Toyozawa, S., Kaminaka, C., Furukawa, F., Nakamura, Y., Matsuoka, H. and Yamamoto, Y. (2012) Chemokine receptor CXCR4 is a novel marker for the progression of cutaneous malignant melanomas. Acta Histochem. Cytochem. 45; 293–299.

41. Yee, C. H., Aoki, S., Uchihashi, K., Matsumoto, A., Yamashita, F., Misago, N., Piao, M., Tetsuji, U., Yonemitsu, N., Sugihara, H., et al. (2010) The air liquid-interface, a skin micro-environment, promotes growth of melanoma cells, but not their apoptosis and invasion, through activation of mitogen-activated protein kinase. Acta Histochem. Cytochem. 43; 1–7.

This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.