Inhibitory Effect of Sodium 5,6-Benzylidene Ascorbate (SBA) on the Elevation of Melanin Biosynthesis Induced by Ultraviolet-A (UV-A) Light in Cultured B-16 Melanoma Cells

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Sodium 5,6-benzylidene ascorbate (SBA) is a conjugate of ascorbic acid (Asc) with benzaldehyde. It has been found that the antioxidant activity of SBA is more stable and has a longer lifetime in living cells and organs than Asc. In this study, we investigated the effect of SBA on the induction of melanin in cultured melanoma (B-16) cells irradiated by UV-A. Melanin content of B-16 cells was significantly increased by UV-A irradiation. The induction was abolished by mannitol and particularly by superoxide dismutase, suggesting the involvement of O_2^- in the biosynthesis of melanin in cultured melanoma cells. This was theorized by the fact that the induction was also observed in B-16 cells treated with superoxide anion radicals chemically generated in the hypoxanthine/xanthine oxidase-reaction system, instead of UV-A irradiation.

The induction of melanin caused by UV-A irradiation was suppressed by SBA in a dose-dependent manner. To elucidate the mechanism of this suppressive effect, the scavenging activity against O_2^- , and the inhibitory effect of SBA on tyrosinase activity were examined. ESR spectrometric analysis showed that SBA strongly scavenged O_2^- , and the presence of SBA in the medium remarkably inhibited the tyrosinase activity in cultured B-16 melanoma cells.

It can be concluded that SBA effectively inhibits the melanin biosynthesis in B-16 melanoma cells induced by reactive oxygen species (ROS) generated by UV-A irradiation *via* tyrosinase.

Key words benzylidene ascorbate; antioxidant; melanin pigmentation; UV-A irradiation; B-16 melanoma cell

Solar ultraviolet (UV) irradiation causes various inflammatory skin conditions such as sunburn, suntan, and so on. Pigmentation on human skin followed by sunburn is thought to be due to overproduction of the dermal melanin. Melanin biosynthesis occurs in melanocytes and involves the oxidation and polymerization of tyrosine to 3,4-dihydroxyphenylalanine (dopa) and dopa to melanin.^{1,2)} In the oxidation of tyrosin, singlet oxygen (1O_2) is believed to cause the intermediate oxidation of dopa to dopachrome and its rapid conversion to melanin.³⁾ Also, tyrosinase is a key enzyme that moves from tyrosin to dopa and dopaquinone. Thus the enzyme tyrosinase is closely related to hyperpigmentation.⁴⁾

Although one of the major functions of melanin in human skin is photoprotection,^{1-3,5)} hyperpigmentation is a serious problem in cosmetics. There have been many reports on agents possessing inhibitory properties against tyrosinase. Ascorbic acid (Asc) is not simply a typical antioxidant, but also an anti-tyrosinase agent. Benzaldehyde (BA), glucosamine and endogenous melanogenic inhibitors have also been reported to have anti-tyrosinase activity.⁶⁾

Sodium 5,6-benzylidene ascorbate (SBA) is a conjugate of Asc with BA. We previously studied the antioxidant activity of SBA in detail and reached the conclusion that the long-lasting activity in living cells was an advantage of this conjugate in comparison with that of Asc.^{7,8)} From another point of view, it is very interesting that both moieties, Asc and BA, of SBA have anti-tyrosinase activity as mentioned above.

In this study, we examined the effect of SBA on melanin biosynthesis in cultured mouse melanoma (B-16) cells induced by UV irradiation from two different viewpoints or antioxidant and anti-tyrosinase activity, and discussed

the possible application of this conjugate as a whitening agent in the cosmetic field.

Chemicals SBA was generously donated by Chemisience Co., Ltd. (Tokyo, Japan). Spin trapping reagents, 5,5-dimethyl pyrrolin-1-oxide (DMPO), and diethylelenetriamine *N,N,N',N'',N'''*-pentaacetic acid (DETAPAC) were purchased from Labotec, Co., Ltd. (Tokyo, Japan). Superoxide dismutase (SOD), catalase (CAT), mannitol (MAN), dimethylfuran (DMF), hypoxanthine (HPX) and xanthine oxidase (XOD) was from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's phosphate buffered saline (PBS) were obtained from Nissui Co., Ltd. (Tokyo, Japan). Tyrosinase (EC 1.14.18.1, >2000 U/mg) from mushrooms was from Sigma Chemical Co., Ltd. (St. Louis, MO, U.S.A.). Other chemicals were purchased from Wako Pure Chemical Industries, Ltd.

Cell Culture The B-16 melanoma cell line was obtained from the Riken Cell bank (Tsukuba, Japan). The cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS, Biowhittaker, Walkersville, Maryland, U.S.A.) at 37 °C in a 5% CO₂/95% air atmosphere. The cells at confluence were harvested with trypsin-EDTA solution (0.1% trypsin/0.01% EDTA) and washed 3 times with Dulbecco's PBS without Ca²⁺ or Mg²⁺ (PBS(-)). The cells (4×10^5) were seeded into 100 mm plastic petri dishes and kept at 37 °C in a CO₂ incubator for 24 h until they reached a monolayer condition.

Ultraviolet-A (UV-A) Irradiation The 24 h-cultured cells were exposed to UV-A light (320–400 nm, Toshiba, FL-20S) at a dose of 0.24 J once a day for 6 d, and the medium was changed just before the exposure at each time interval. The radical scavengers, SOD (1000 U/ml), CAT (200 U/ml), MAN (100 μM), and DMF (100 μM) were

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involved in the medium.

Assay of Melanin Content in Cultured B-16 Melanoma Cells The cultured cells were harvested with 1% trypsin–0.01% EDTA solution, and suspended in ice-cold PBS(–) solution. Cells were washed 3 times with ice-cold PBS(–) by centrifugation at 3000 rpm for 3 min. The cell pellets (3×10^6 cells/tube) were kept in 5% trichloroacetic acid (TCA) solution for 30 min at 0 °C, and then rinsed 3 times with ethanol and ether. The melanin was dissolved in solune from the residue. Optical density of the solution was measured at 400 nm. The melanin content was expressed as OD/10⁶ cells.

Assay of Tyrosinase Activity Aliquots of the washed cells cultured with and without SBA were subjected to assay for tyrosinase activity. Briefly, the cells were suspended in PBS(–) containing 0.1% Triton X-100, homogenized with a sonicator (Handy Sonic UR-20P, Tomy Seiko Co., Ltd., Tokyo, Japan), and centrifuged at 10000 rpm for 30 min. The supernatant was assayed for tyrosinase activity. The activity was measured spectrophotometrically at 475 nm according to the method of Pomerantz⁴⁾ with a slight modification. The reaction mixture contained 0.4 ml of supernatant and 0.4 ml of L-dopa (0.5 mg/ml) in 0.1 M phosphate buffer (pH 6.8). The L-dopa solution was preincubated for 10 min at 37 °C and the reaction was started by adding the supernatant solution. The change in absorption at 475 nm following the oxidation of L-dopa to dopachrome was measured during the first 5 min. Specific activity was expressed as U/l calculated from $\Delta E/\text{min}$ at 475 nm.

Effect of SBA on Tyrosinase Activity Effect of SBA on tyrosinase activity was examined in a cell-free system. The reaction mixture contained 0.8 ml of 50 mM phosphate buffer (pH 6.8), 0.5 ml tyrosinase (Boehringer Yamanouchi Co., Tokyo, Japan; 1.25 U/ml), 0.5 ml L-dopa (0.5 mg/ml), and 0.2 ml of varying concentrations of SBA. The reaction was started by adding the tyrosinase to the preincubated L-dopa solution with and without SBA.

Analysis of the Scavenging Activity of SBA against $\cdot\text{O}_2^-$ and $\cdot\text{OH}$ Superoxide anion radicals were generated from the HPX/XOD-reaction system. The reaction was started by adding 0.25 U/ml of XOD to 75 mM PBS (pH 7.8) containing 0.5 mM HPX, 0.96 mM DETAPAC and 0.69 mM DMPO with and without an appropriate concentration of SBA, and then the reaction mixture was quickly put into an ESR quart flat cell. The cell was placed in the cavity of the ESR spectrometer, and the ESR spectrum was recorded exactly 1 min after adding XOD. ESR spectra were recorded on a JEOL X-band spectrometer, Model JES-FR80, at 100 kHz magnetic field modulation using an aqueous quartz flat cell (inner size 60 mm $\times$ 10 mm $\times$ 0.31 mm) with an effective sample volume of 160 μl . The magnetic field was 335 ± 5 mT, microwave power 8 mW, amplitude 5×10^2 , modulation amplitude 0.02 mT, response time 0.1 s, and sweep time 2 min. Hydroxyl radicals were generated from the Fenton-reaction. The reaction was started by adding 1 mM hydroperoxide to 75 mM PBS (pH 7.8) containing 50 mM ferrous sulfate, 100 mM DETAPAC and 10 mM DMPO with and without SBA. The spectra were obtained in the

same way as those in superoxide anion radicals.

Induction of Melanin by O_2^- Exposure B-16 melanoma cells (4×10^5) were seeded into 100 mm plastic petri dishes and cultured for 24 h under a humidified atmosphere of 5% CO_2 in air and then exposed to superoxide anion radicals generated by a HPX (0.25 mM)/XOD (50 mU)-reaction system for 20 min. The exposed cells were washed 3 times with ice-cold PBS(–), and then cultured for 6 d. The medium was changed at once a day.

Statistical Analysis Results were analyzed for statistical significance using the Student's *t* test for unpaired observations.

RESULTS

The induction of melanin in B-16 cultured cells by UV-A irradiation was first examined. As can be seen in Fig. 1, the melanin content in the cells was significantly raised by UV-A irradiation ($p < 0.001$).

To clarify reactive oxygen species (ROS) involved in the induction of melanin by UV-A irradiation, the effects of

Fig. 1. Effect of UV-A Irradiation on Melanin Biosynthesis in Cultured B-16 Melanoma Cells

B-16 cells were exposed to UV-A irradiation at a dose of 0.2 J/d for 6 d. Each value is indicated as the mean $\pm$ S.E.M. of 3 independent determinations. *** Significantly different from the UV-A-non-irradiated group at $p < 0.001$.

Fig. 2. Effect of Radical Scavengers on Melanin Biosynthesis in Cultured B-16 Melanoma Cells Induced by UV-A Irradiation

Cells were exposed to UV-A irradiation in the presence of radical scavengers including SOD (1000 U/ml), CAT (200 U/ml), MAN (100 μM), and DMF (100 μM). Each value is expressed as relative percent with respect to the UV-A-irradiated group, and is the mean $\pm$ S.E.M. of 3 determinations. **, *** Significantly different from the UV-A-irradiated group at $p < 0.01$, and $p < 0.001$.

Fig. 3. Effect of Superoxide Anion Radical Exposure on Melanin Biosynthesis in Cultured B-16 Melanoma Cells

Superoxide anion radicals were generated from the HPX/XOD reaction system, and the cells were exposed to the radicals on the first day and thereafter cultured for 6 d. Each value is indicated as relative percent with respect to the UV-A-irradiated group and is the mean $\pm$ S.E.M. of 3 determinations. ** Significantly different from the UV-A-non-irradiated group at $p < 0.01$.

Fig. 4. Effect of SBA on Melanin Biosynthesis in Cultured B-16 Melanoma Cells Induced by UV-A Irradiation

The cells were exposed to UV-A irradiation with different concentrations of SBA. Each value is expressed as relative percent with respect to the UV-A-irradiated group and is the mean $\pm$ S.E.M. of 3 determinations. ** Significantly different from the UV-A-irradiated group at $p < 0.01$.

different kinds of scavengers on the melanin induction were determined. The induction was significantly suppressed by MAN and particularly by SOD, a scavenger for superoxide anion radicals (Fig. 2), while neither DMF nor CAT, respective scavengers for singlet oxygen and hydrogen peroxide, significantly modified the induction of melanin.

These data suggested that superoxide anion radical may primarily mediate the increase of melanin biosynthesis in B-16 melanoma cells followed by UV-A irradiation. The role of superoxide anion radical in melanin biosynthesis was supported by the fact that this molecule alone increased the biosynthesis to a degree equal to that on UV-A irradiation (Fig. 3).

Investigation of the effect of SBA on the rise in melanin biosynthesis induced by UV-A irradiation showed that SBA significantly inhibited the higher melanin content in

Fig. 5. Effect of SBA on the Elevated Tyrosinase Activity in Cultured B-16 Melanoma Cells Induced by UV-A Irradiation

The cells were exposed to UV-A irradiation with or without different concentrations of SBA. Each value indicates the mean $\pm$ S.E.M. of 3 independent assays. *, ** Significantly different from the irradiated group at $p < 0.05$ and $p < 0.01$, respectively.

Fig. 6. Effects of SBA, Asc and BA on Tyrosinase Activity in Cell-Free System

The value represents percent inhibition at each concentration and is the mean of three determinations.

a dose-dependent manner (Fig. 4). We also assayed the tyrosinase activity, a key enzyme of melanin biosynthesis in melanocytes, in the UV-A irradiated B-16 melanoma cells cultured with and without SBA. As can be seen in Fig. 5, UV-A irradiation significantly raised this activity ($p < 0.05$). This elevation was significantly suppressed by SBA in a similar fashion as in melanin biosynthesis. In addition, the activities in the cells cultured with SBA at concentrations of over 0.1 mM were even lower than those of non-irradiated cells. SOD caused the activity to remain at almost the same level as UV-A-non-irradiated cells, suggesting that tyrosinase may be induced by stimulation of the superoxide anion radical.

The scavenging activity of SBA against superoxide anion and hydroxyl radicals, which are both thought to have important roles in melanin biosynthesis, was inves-

tigated using ESR spectrometry. The superoxide anion radicals were generated by HPX/XOD reaction system in the presence of DMPO with or without SBA. SBA suppressed the formation of DMPO-OOH in a dose-dependent manner. It also interfered with the reaction of hydroxyl radicals generated by the Fenton reaction with DMPO. The IC_{50} values of SBA were about 25 and 80 μM , respectively.

The inhibitory effects of Asc and BA are already well recognized.⁶⁾ We have also studied the efficacy of SBA which is composed of Asc and BA on tyrosinase activity in the cell-free system. As shown in Fig. 6, SBA showed a strong anti-tyrosinase activity, even though it was only half that of free Asc at 100 μM . The activity of BA was far less than those of Asc and SBA.

DISCUSSION

It is well known that UV irradiation causes various inflammatory skin conditions such as sunburn and suntan, and is often related to the development of skin cancer *via* DNA damage.⁹⁻¹³⁾ In molecular photodermatology, UV induces ROS in the epidermis and these highly reactive molecules are thought to have important functions in the pathogenesis of abnormalities as described above.

Pigmentation developed after the sunburn is believed to be due to the melanin accumulation in epidermis. ROS generated by UV irradiation may, in part, be responsible for the melanin biosynthesis in melanocytes.^{14,15)} Briefly, melanin is a polymer complex formed by various kinds of tyrosine-related metabolites like dopaquinone and indolequinone. These metabolites are produced by oxidation of tyrosine in the presence of ROS; singlet oxygen is thought to probably contribute to the beginning of the oxidation reaction.³⁾

The major function of melanin in human skin is believed to be as a filter which absorbs the radiation energy in the UV, thus protecting the skin from inflammatory damage.¹⁻³⁾ Melanin also possesses SOD-like activity to scavenge superoxide anion radicals.⁵⁾ Apart from these important roles of melanin pigment, however, overproduction of melanin leading to hyperpigmentation poses a serious problem for a cosmetic beauty, and many anti-drugs having inhibitory potency against melanin accumulation have been researched. Two different mechanisms for the action of anti-drugs have been proposed¹⁶⁻¹⁹⁾; one is the inhibitory action against melanin biosynthesis, and the other is the action of cytotoxicity for melanocytes. The former drugs with direct inhibitory potency on tyrosinase have been of greatest interest: Asc, BA, glucosamine and endogenous melanogenic inhibitors are now well known.⁶⁾

SBA can be a very interesting compound from the following viewpoints: Asc moiety of SBA has an excellent antioxidant activity against ROS including superoxide anion radical, hydroxyl radical, and lipid peroxide. Also, this conjugate is composed of Asc and BA which both effectively inhibit tyrosinase activity.

We have focused our attention on antioxidant activity in this study, and examined the effect of SBA on melanin pigmentation. We previously reached the conclusion that

a more stable and longer lifetime activity in living cells was an advantageous factor in SBA anti-oxidant activity in comparison with free Asc. This was further confirmed by its excellent protecting potency on the lipid peroxidation of phosphatidylcholin-liposomes induced by UV irradiation exposure (data not shown).

Induction of the melanin pigment by UV-A irradiation exposure was first investigated in cultured B-16 melanoma cells. UV-A irradiation induced a significant elevation of melanin content to around 1.5 times that of the non-irradiated group. Involvement of ROS in melanin biosynthesis has been reported; singlet oxygen has been reported to be an initiator of melanin biosynthesis.²⁾ The induction, however, was remarkably abolished by SOD under our experimental conditions, suggesting that superoxide anion radical generated by UV-A irradiation may be involved in the melanin biosynthesis. This speculation is supported by the fact that the melanin biosynthesis was induced by superoxide anion radical exposure chemically generated in the HPX/XOD reaction system as well as UV-A irradiation. Based on these data, we can further suggest the role of superoxide anion radical as a signal transmitter for melanin biosynthesis, and speculate that SBA blocks the induction of melanin biosynthesis because of its strong scavenging activity against superoxide anion radical. As can be seen Fig. 3, SBA inhibited the elevation of melanin content induced by UV-A irradiation dose-dependently.

In melanin pigmentation, tyrosinase is a key enzyme of melanin biosynthesis as described above, and it is of interest whether or not UV-A irradiation induces tyrosinase. As can be seen in Fig. 5, the UV-A irradiation actually increased the tyrosinase activity in the cultured B-16 melanoma cells; the degree of induction appeared to accord well with that of melanin content. The induction was also significantly suppressed by SBA in the same pattern as in melanin content. This suggests that superoxide anion radical may act in the induction of tyrosinase, being closely related to melanin biosynthesis as an inducer. This is probably supported by the fact that the induction of tyrosinase induced by UV-A was also completely abolished by SOD. The increase of tyrosinase activity by the exposure to O_2^- generated by the HPX/XOD reaction system has been confirmed (data not shown). It is noteworthy that the activity was suppressed to an even lower level than that of the non-irradiated group; this is probably due to the original tyrosinase of non-irradiated B-16 melanoma cells themselves being inhibited by SBA, since its inhibitory potency remained (Fig. 6). From these data, it is suggested that the increase of melanin content in cultured B-16 cells was resulted, in part, from the induction of tyrosinase by O_2^- followed by UV-A irradiation exposure.

The cytotoxicity of SBA on the cells should also be viewed as another mechanism for its inhibitory efficacy on melanin biosynthesis. However, the inhibitory effect of SBA by killing melanocytes can be ignored, because of its significantly low cytotoxicity; also, the cell viability under our experimental conditions was usually more than 95%.

We would also like to mention that SBA was effective in protecting against on a delayed type of erythema on

guinea pig skin caused by UV-A irradiation exposure (data not shown).

In conclusion, we found that UV-A irradiation causes the induction of melanin by stimulating tyrosinase with O_2^- , and that SBA effectively blocks this stimulation, thus inhibiting the melanin biosynthesis in cultured B-16 melanoma cells. These data suggest the potential usefulness of SBA as a therapeutic drug in hyperpigmentation.

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