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Blue light inhibits melanin synthesis in B16 melanoma 4A5 cells and skin pigmentation induced by ultraviolet B in guinea-pigs

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Background: **Little has been known about the effects of visible light in mammalian cells. We recently found that blue light not only suppressed the growth of B16 melanoma cells in a time-dependent manner but also inhibited metastasis of the B16 melanoma cells to the lung. These findings suggest that exposure to blue light modifies the functions of B16 melanoma cells. The present study investigated the effects of blue light on B16 melanoma 4A5 cells and Weiser–Maple guinea-pigs to confirm the biological effect of blue light on melanin formation.**

Method: **The effect of red, green, and blue light on melanin synthesis in B16 melanoma 4A5 cells was measured. The back skin of brown Weiser–Maple guinea-pigs was exposed to ultraviolet B (UVB; 588 mJ/cm^2 (0.7 mW/cm^2 × 14 min) three times a week for 2 weeks to induce melanin deposition. Thirty minutes after each UVB exposure, blue light was applied for 30 min. Pigmentation of the exposed areas of skin was checked once a week, and photographs of the skin were taken by digital camera. Observation was continued for 18 days after the final UVB exposure.**

Result: **Melanin synthesis in B16 melanoma 4A5 cells was selectively suppressed by blue light, but blue light did not induce decolorization of previously produced melanin. In the back skin of brown guinea-pigs, the brightness of the sites exposed to UVB began to decrease on the fifth day of the experiment, decreasing further from the 12th day to the 18th day after UVB exposure. The brightness of the sites exposed to UVB and blue light decreased in a manner similar during the UVB exposure, but remained relatively unchanged from the 12th day to the 30th day.**

Conclusion: **These results suggest that blue light suppresses melanin formation following repeated UVB exposure. Further investigation with various light such as blue light may lead to a new approach to the care of ultraviolet-affected skin such as hyperpigmentation.**

Key words: **blue light; guinea-pig; LED; melanin; UVB.**

Numerous studies have been carried out on the relationship between light and diseases, with special reference to the application of radiation and ultraviolet rays (UV) for cancer therapy (1–5) and for the evaluation of carcinogenic (6–10) and immunosuppressive (11) effects of light. Little attention has been paid to the effects of visible light in medical application. We recently examined the effects of components of visible light on the growth of cancer cells using light-emitting diodes (LEDs), which have specific light-emission spectra, and found that blue light suppressed the growth of B16 melanoma cells in a time-dependent manner (12). We suggested that this effect of blue light could be due to the inhibition of DNA synthesis and cell division. When the blood of rats with 1-ethyl-1-nitrosourea-induced leukemia was exposed to blue light for 3 h during extracorporeal circulation, the growth of leukemic cells was suppressed, but the growth of normal lymphocytes was not affected significantly (13). Additionally, when B16 melanoma cells exposed to blue light and incubated for 7 days were injected intravenously into mice, metastasis of the B16 melanoma cells to the lung was significantly inhibited (14). The suppressing effect of blue light on the proliferation of B16 melanoma cells continued to be present even after the passage of cells over several generations (unpublished data), and exposure to blue light resulted in marked alterations

in morphology of the cell colonies, with the formation of relatively low-density colonies of long and large striated cells (12). These findings suggest that exposure to blue light modifies the functions of B16 melanoma cells. Melanin-producing capacity is one of the main functions of B16 melanoma cells. In this study, we examined the effects of blue light on the melanin-producing capacity of B16 melanoma 4A5 cells (a melanin-producing cell line) and Weiser–Maple guinea-pigs (a melanin-producing species) to confirm the biological effect of blue light on melanin formation.

Materials and methods

LED and UV-irradiation apparatus

The irradiation apparatus were constructed using red, green, and blue LEDs (Nichia, Tokushima, Japan) as reported previously (12). Each LED was driven by direct current from a standard power supply (S82K-10024, Omron, Tokyo). The effects of the three primary colors on melanin production *in vitro* were evaluated as described below. Specifications of the LEDs were: current, wavelength, and irradiance of 32.0 mA, 634 nm, and 2.27 mW/cm^2, respectively, for the red LED; 30.5 mA, 518 nm, and 2.26 mW/cm^2, respectively, for the green LED; and 8.0 mA, 470 nm, and 2.24 mW/cm^2, respectively, for the blue LED. In the experiments designed to evaluate the direct effects of blue light on extracted melanin and the inhibitory action of blue light on the melanin deposition induced by UVB irradiation in brown guinea-pigs, the blue LED used had the following specifications: current 30.1 mA, wavelength 470 nm, and irradiance 5.7 mW/cm^2. For UVB exposure to guinea-pig skin, UV light in the wavelength range of 280–360 with a peak at 305 nm was used (FL20S E30, Toshiba Medical Supply, Tokyo, Japan). Intensity of exposure was measured with a UV radiometer (UVR-305/365 D (II), Tokyo Kogaku Kikai KK, Tokyo, Japan). UVB exposure was conducted at a dose of 588 mJ/cm^2/day (0.7 mW/cm^2 × 14 min/day).

Cell culture

B16 melanoma 4A5 cells (RCB0557, supplied by Riken Cell Bank, Tsukuba, Japan) cultured in Dulbecco's modified Eagle's medium (D5796, Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (FBS, Life Technologies, Grand Island, NY) were used for the experiments. They were grown for three cell divisions to stabilize the growth before experiments. Cells were grown at 37 °C in 5% CO_2/95% air atmosphere.

Effects of each component of visible light on melanin synthesis of B16 melanoma 4A5 cells

Cell suspensions (15 ml) containing 3×10^3 to 3×10^4 cells/ml were inoculated into incubation dishes (Omnitray 165218, Nunc, Naperville, IL). After 24 h of inoculation, cells were exposed to red (2.27 mW/cm^2), green (2.26 mW/cm^2), or blue (2.24 mW/cm^2) light for 45–47 min, followed by further incubation for 4–8 days. The controls were treated in the same manner except for light exposure. After the incubation, cells were counted by trypan blue staining and suspended in SOLUENE-350 (PerkinElmer Life Sciences, Boston, MA) at 1.2–1.5 × 10^6 cells/ml. The melanin-producing capacity of the cells was measured by the light absorbance at 405 nm.

Effects of blue light on previously produced melanin in B16 melanoma 4A5 cells

After incubation for several days, B16 melanoma 4A5 cells (3×10^6–10^7 cells) were suspended in 0.5 ml of SOLUENE-350. After dilution with toluene, 0.3 g of the suspension was placed in a glass vial and exposed to blue light (5.7 mW/cm^2) for 1–1.5 h/day for 3 days. The suspension was then allowed to stand for 2 days and supplemented with toluene for evaporation during the experiment, followed by measurement of absorbance at 405 nm.

Experimental animals

Six 5-week-old male Weiser–Maple guinea-pigs were purchased from Kiwa Experimental Animal Laboratories (Wakayama, Japan) and used for the experiments when they were 6 weeks of age. Experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals established by the Science Council of Japan.

Exposure of guinea-pigs to UVB and blue light

The back skins of guinea-pigs were depilated. The experimental schedule is shown in Fig. 1. Four test sites (two anterior and two posterior regions of the back) were marked on the back of each animal, and these sites were exposed to UVB of 588 × mJ/cm^2 (0.7 mW/cm^2 × 14 min). Thirty minutes after UVB exposures, two test sites (right hand anterior and posterior regions of the back) were exposed to blue light (5.7 mW/cm^2) for 30 min (UVB plus blue). Before the exposure to blue light, two other sites (left-hand anterior and posterior regions of the back) were covered with gauze secured with adhesive elastic bandages made of brown cloth to block the blue light (UVB only). Both UVB and blue light were applied three times a week (every other day) for 2 weeks, and

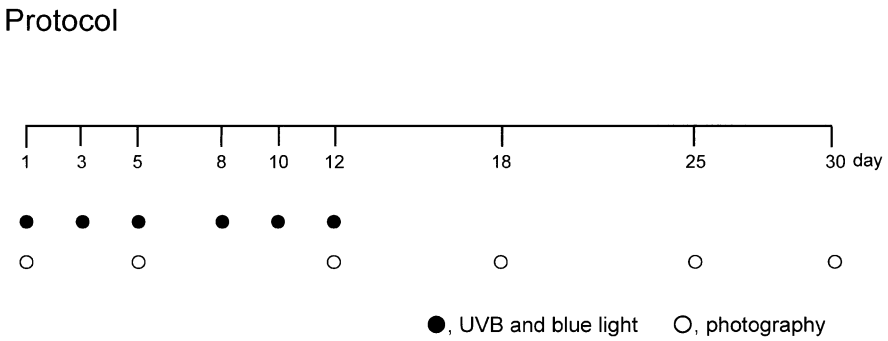

Fig. 1. Experimental design for UVB and blue-light exposure to guinea-pigs and photography of the effect. Day 1: before exposure to UVB.

observation was made each time in six animals. The back skin of each animal was checked for pigmentation and photographed with a digital camera.

Observation and testing of the skin

Pigmentation of the exposed areas of skin was checked once a week, and photographs of the skin were taken by digital camera. Observation was continued for 18 days after the final UVB exposure. The photographs were fed into an image-processing computer to obtain images of the pigmented areas. Images of the regions of interest were saved in Windows bitmap format. Then, using Scion Image for Windows (Scion, Frederick, MD), histograms of the frequency of each of 256 tones were prepared for the relative numerical conversion of the images.

Statistical analysis

The results are expressed as mean ± SD. *F*-test was applied to detect differences in the variance between two groups. If the variance was homogeneous, Student's *t*-test was employed; otherwise, the Aspin Welch *t*-test was used. For comparisons among three or more groups, Dunnett's test was applied.

Results

Effects of each component of visible light on melanin synthesis in B16 melanoma 4A5 cells

Incubation of B16 melanoma 4A5 cells resulted in the release of black pigment into the medium. Proliferation of the B16 melanoma 4A5 cells was suppressed in the blue-light-exposed group, but was not suppressed in the red- or green-light-exposed groups (Fig. 2). Melanin concentration in the same number of cells was found to be markedly lower in the blue-light-exposed group as compared with that in the control group (Figs 3 and 4). However, blue light did not induce decolorization of the extracted melanin solution even after exposure to blue light for 3 days (1–1.5 h/day), as shown in Fig. 5.

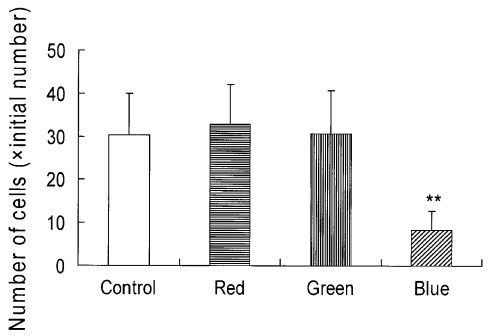

Fig 2. Number of cells after 4–8 days of exposure to red, green, or blue light. Data are expressed as mean ± SD ($n = 11$). $^{**}P < 0.01$ vs. control.

Skin pigmentation and image analysis

None of the test animals showed any abnormalities throughout the experiments. Both the UVB-exposed and the UVB- and blue-light-exposed sites were without skin inflammation, such as erythema and desquamation. Figure 6 shows pigmentation of the skin areas of the guinea-pigs exposed to UVB and UVB plus blue light. Figure 7 shows the results of image analysis by digital camera photographs. Five days after the start of exposure, skin pigmentation began to be noted, and the brightness of the skin as measured by image analysis decreased. In all animals, the rate of pigmentation differed between skin on the anterior and posterior region of the back, with more intense pigmentation in the anterior region of the back. At the UVB-only sites, pigmentation increased in both the anterior and posterior regions of the back from the fifth day, the first measurement after the onset of UVB exposure. On the 12th day of the experiment, the brightness level decreased from 129 to 113 in the anterior region of the back skin and from 134 to 123 in the posterior region of the back skin. In

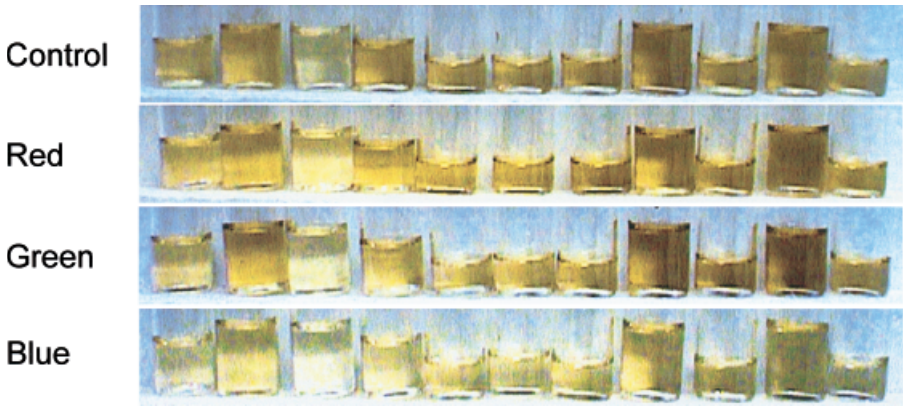

Fig 3. Photographs of melanin produced after exposure to red, green, or blue light. After the procedure mentioned for Fig. 2, cells were suspended in SOLUENE-350 at 1.2–1.5 $\times$ 10^6 cells/ml. Four melanin-containing solutions (vertically shown in the figure) represent a set of one experiment. A total of 11 experiments were performed with differing cell culture conditions ($n = 11$ in each group). When the concentration of melanin was too low to measure light absorbance, we decreased the volume of solvent (SOLUENE-350). The number of B16 melanoma 4A5 cells in each group was synchronized to the same level.

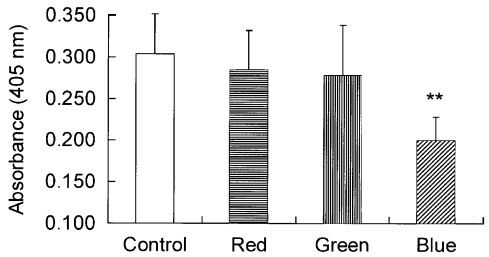

Fig 4. Melanin production after 4–8 days of exposure to red, green, or blue light. After the procedure mentioned for Figs 2 and 3, light absorbance at 405 nm of melanin-containing solutions in Fig. 3 was measured for the determination of melanin production. Data are expressed as mean $\pm$ SD ($n = 11$). $^{**}P < 0.01$ vs. control.

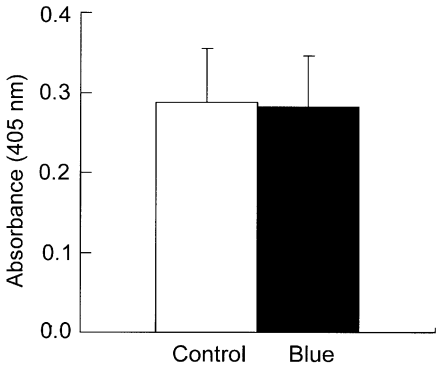

Fig 5. Effect of blue light on previously produced melanin. After incubation for several days, B16 melanoma 4A5 cells (3×10^6–10^7 cells) were suspended in SOLUENE-350 at 1.2–1.5 $\times$ 10^6 cells/ml and the suspension was diluted with toluene to adjust the total weight of the suspension to 0.3 g. Blue light was applied for 1–1.5 h/day for 3 days. The suspension was then allowed to stand for 2 days and supplemented with toluene to make up for evaporation, and light absorbance at 405 nm was measured to measure the amount of melanin. Data are expressed as mean $\pm$ SD ($n = 10$).

the anterior region of the back, skin pigmentation continued to increase even after the final UVB exposure. On the 18th day of the experiment (6 days after the final UVB exposure), the brightness level in this area had decreased to 101 and remained relatively unchanged from the 18th day through the 30th day. In the posterior region of the back, the brightness level remained relatively unchanged after the 12th day. In the UVB- and blue-light-exposed sites, changes in the level of brightness followed a course similar to that at the control sites until the 12th day, with a decreased brightness level in the skin on the anterior region of the back on the 12th day. From the 12th day to the 18th day, little enhanced pigmentation was noted at the UVB- and blue-light-exposed sites, unlike that seen at the UVB-only sites. The brightness level of the skin on the anterior region of the back at UVB- and blue-light-exposed sites was 115 on the 18th day, similar to the level recorded on the 12th day. Thus, progression of the pigmentation on the anterior region of the back was significantly suppressed at the UVB plus blue-light-exposed sites, and the brightness of this area remained relatively unchanged after the 18th day. The skin on the posterior region of the back showed a decrease at the UVB plus blue-light-exposed sites, but the progression of pigmentation was slower than that in the skin on the anterior region of the back, similar to the findings at the corresponding UVB-only sites. On the 12th day, however, the brightness level of the skin at the UVB

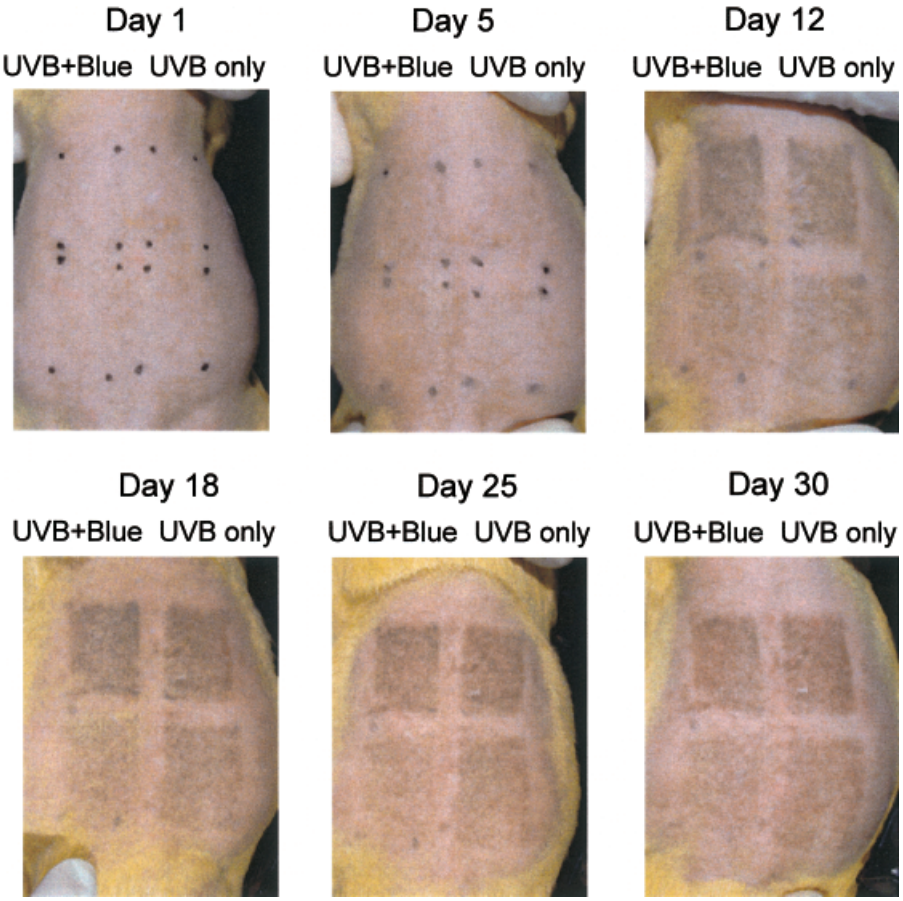

Fig 6. Photographs of the skin after exposure to UVB with or without blue light. Four test sites (two anterior and two posterior regions of the back) were marked on the back of each animal, and these sites were exposed to UVB of 588 mJ/cm^2 (0.7 mW/cm^2 × 14 min). Thirty minutes after UVB exposures, two test sites (right-hand anterior and posterior regions of the back) were exposed to blue light (5.7 mW/cm^2) for 30 min (UVB plus blue). Before the exposure to blue light, two other sites (left-hand anterior and posterior regions of the back) were covered with gauze secured with adhesive elastic bandages made of brown cloth to block the blue light (UVB only). Both UVB and blue light were applied three times a week (every other day) for 2 weeks, and observation was made each time in six animals.

plus blue-light sites on the posterior region of the back was 130, indicating significant suppression of pigmentation as compared with that at the UVB-only sites, where the brightness level was 123. After the 12th day, brightness of the skin on the posterior region of the back remained almost unchanged in both sites.

Discussion

Among red, green, and blue light, only blue light selectively suppressed melanin synthesis in B16 melanoma 4A5 cells. Since blue light did not induce any decolorization of the extracted melanin, the reduction in melanin levels in B16 melanoma 4A5 cells following blue light exposure does not represent a direct effect on the existing melanin, and may indicate the suppression of melanin synthesis in the cells. We used Dulbecco's modified Eagle's medium for the culture medium of B16 melanoma 4A5 cells because of optimal growth. We recently found that riboflavin augments the effect of blue light inhibition of the growth of B16 melanoma cells by producing free radicals, and this effect was reversed by catalase (15). Since the medium contains 1.0 μM riboflavin – a photosensitizer – free radicals generated after exposure to blue light could inhibit cell growth and melanin production. Another possibility that low-density cultures after blue light exposure might produce fewer melanin than high-density cultures is conceivable. The detailed mechanism of this phenomenon including photoproduct formation and analysis of melanin-synthesizing enzyme (tyrosinase, etc.), remains to be elucidated.

It is known that skin pigmentation is the result of synthesis of melanin induced by the activation of enzymes (tyrosinase, etc.) in melanocytes in the basal

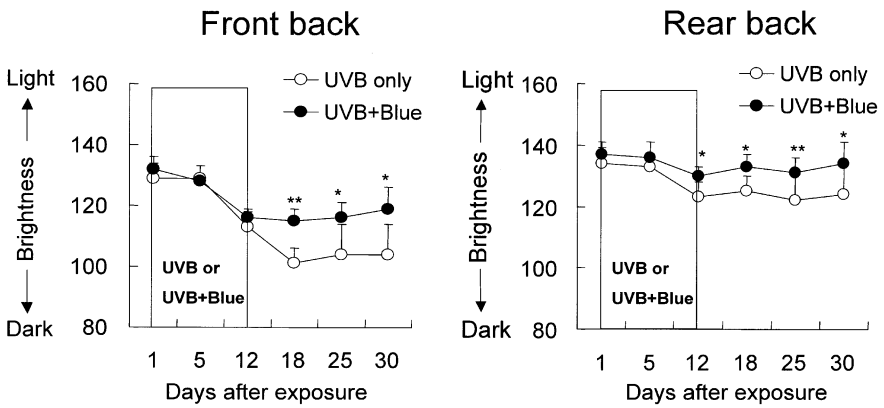

Fig 7. Melanin pigmentation of the skin after exposure to UVB with or without blue light. Photographs of the skin were taken with a digital camera once a week until the 18th day after the final UVB exposure. The photographs were processed to measure brightness by calculating the frequency of each of 256 tones for numerical conversion of the images. Data are expressed as mean ± SD ($n = 6$). *$P<0.05$, **$P<0.01$ vs. control.

layer of the epidermis in response to factors such as UV light. The back skin of brown guinea-pigs, which is capable of producing melanin in the same manner as humans, was exposed to blue light in order to examine its effects on the formation and deposition of melanin. Skin brightness decreased linearly after the second UVB exposure (Fig. 7). It seems likely that repeated exposure to UVB activated the melanocyte enzymes involved in the biosynthesis of melanin, leading to a marked increase in melanin formation. Brightness of the skin on the anterior regions of the back continued to decrease for 6 days after the final UVB exposure, while the posterior region of the back skin remained relatively unchanged after the final UVB exposure. These results may be due to either differences in the sensitivity of the melanocytes to UV light or differences in cell density between the skin on the anterior and the posterior region of the back. We assume that the formation of melanin by activated enzymes in the skin on the anterior region of the back and its deposition in the skin continued to be active even after the end of UVB exposure and exceeded the amount of pigment lost by desquamation of the epidermis.

The brightness level in the anterior region of the back skin did not differ significantly between the UVB-only and UVB plus blue-light-exposed sites during the UVB exposure period, but the latter sites showed less significant a decrease in the brightness level as compared with that at the UVB-only sites when measured 6 days after the final UVB exposure. In the posterior region of the back skin, decrease in skin brightness at the UVB plus blue-light-exposed sites after the final UVB exposure was only slightly suppressed. These results suggest that the effect of blue light appears to be manifested when melanin formation in melanocytes has been adequately activated by repeated UVB exposure, rather than in the initial stages when melanocytes have not yet been sufficiently activated. Although hyperpigmentation induced by photosensitizer plus visible light was reported (16), blue light only inhibited hyperpigmentation induced by UVB in this study. These results suggest that the amount of photoproduct produced by blue light was not enough to induce hyperpigmentation in guinea-pig skin. The possibility that blue light inhibits UVB-induced photo-damage should be examined.

It has recently been shown that stratospheric ozone depletion increases the exposure of humans to UV light from the sun, resulting in the enhanced incidence of skin cancer (8–10) and immunosuppression (11, 17). Ultraviolet light has also been attracting close attention for esthetic reasons, because it stimulates the deposition of melanin in the skin and is responsible for hyperpigmentation, nevi and sunburn. We recently found that blue light inhibits the incidence rate and the number of papillomas in the v-Ha-ras transgenic mouse (18). Further investigation including long-term treatment of skin with various light such as blue light, may lead to a new approach to the care of UV-affected skin such as hyperpigmentation.

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