Jpn. J. Cancer Res. **93**, 551–558, May 2002

# Blue Light Inhibits the Growth of B16 Melanoma Cells

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**Although a number of studies have been carried out to examine the biological effects of radiation and ultraviolet radiation (UV), little is known concerning the effects of visible light. In the present study, exposure of B16 melanoma cells to blue light (wavelength 470 nm, irradiance 5.7 mW/cm$^2$) from a light-emitting diode (LED) inhibited cell growth in proportion to the period of exposure, with no increase observed in the number of dead cells. The number of B16 melanoma colonies that formed after exposure to blue light for 20 min was only slightly less than that in non-exposed controls, but the colony size as assessed by the area covered by colonies and cell counts per colony were markedly decreased. The percentages of G0/G1 and G2/M phase cells were markedly increased, with a reduction in S phase cells as determined by flow cytometry after exposure to blue light. Furthermore, analysis of the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA also showed a reduction in the percentage of S phase cells after exposure. These results indicate that blue light exerts cytostatic effects, but not a cytocidal action, on B16 melanoma cells.**

Key words: Blue light — LED — B16 melanoma cell — Cytostatic effect — Cytocidal action

Numerous studies have been performed to investigate the relationships between light and disease, with special attention paid to the application of radiation and UV to cancer treatment[1–5] and the evaluation of carcinogenic[6–10] and immunosuppressive[11] effects. In recent years, photodynamic therapy for cancer using photosensitive substances and lasers has been increasingly applied in clinical medicine.[12] On the other hand, the number of studies concerning the direct effects of visible light is quite small in the medical field as compared to, for example, the field of botany. Although the effect of visible light on humans has been examined with respect to the circadian clock,[13] the retina,[14, 15] and hyperbilirubinemia in infantile jaundice,[16, 17] little is known concerning the effects of visible light on cancer cells. With regard to effects on cell-proliferation processes, although it has been reported that *p53* is induced by UV or γ-radiation[18, 19] and that *p53* or genes related to the cell cycle are rapidly and selectively induced after DNA lesions, leading to restoration of DNA or cell death,[20] the effects of visible light have not yet been clarified.

One reason for the small number of such studies has been the lack of availability, until recently, of compact, low-cost devices for emitting light in specific ranges of the visible spectrum. In 1993, Nakamura *et al.* developed the first commercially available ultrahigh-luminance blue light-emitting diode (LED), made of gallium nitride.[21] This was followed by the introduction of practical blue-green and green LEDs in 1995.[22] Thus, the availability of these newly developed compact blue and green LEDs with very low power consumption and low heat generation, in addition to red LEDs, which have been available for many years, has recently made it possible to develop an apparatus that can emit the three primary colors of the visible spectrum.

The present study was undertaken to examine the effects of visible light emitted by red, green, and blue LEDs on the growth of B16 melanoma cells.

## MATERIALS AND METHODS

**LED- and UV-irradiation apparatus** The apparatus was constructed using red, green, and blue LEDs (Nichia Corp., Tokushima) and designed to fit into standard 96-well microplates used for cell culture (Fig. 1). Each LED was driven by direct current from a standard power supply (S82K-10024, Omron, Tokyo). The LEDs had the following specifications: red=current 32.0 mA, wavelength 634 nm, and irradiance 2.9 mW/cm$^2$; green=31.6 mA, 518 nm, and 2.3 mW/cm$^2$; and blue=30.1 mA, 470 nm, and 5.7 mW/cm$^2$. For UVC irradiation, a Toshiba GL15 (wavelength 253.7 nm and irradiation intensity 0.13 mW/cm$^2$; Toshiba Corp., Tokyo) was used.

**Cell culture** B16 melanoma cells (JCRB0202, Human Science Research Resources Bank, Osaka) cultured in Eagle's minimum essential medium (MEM) (Nihon Seiyaku, Tokyo) with 10% fetal bovine serum (FBS, Life Technologies, Inc., Grand Island, NY) were used in the experiments when their growth rates had stabilized after cultivation for three generations. Cells were incubated at 37°C in a 5% $CO_2$/95% air atmosphere.

**Growth inhibition of B16 melanoma cells** Cell suspen-

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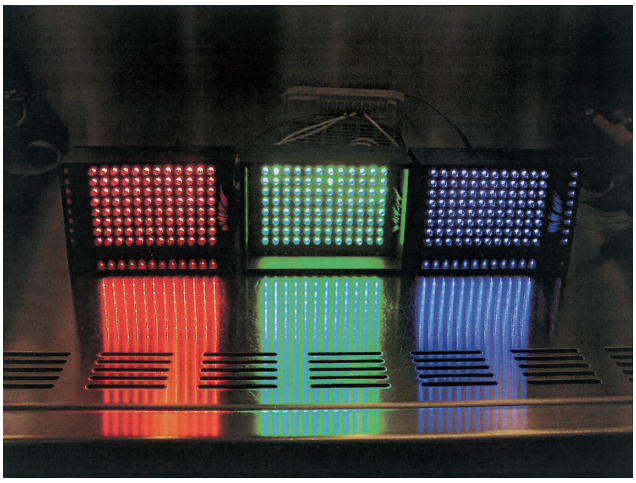

Fig. 1. LED apparatus used to emit light of three primary colors (red, wavelength 634 nm, green, 518 nm and blue, 470 nm).

sions (100 $\mu$l) containing $3\times10^3$ to $1\times10^5$ cells/ml were inoculated into 96-well microplates (Becton Dickinson, Franklin Lakes, NJ). At 24 h after inoculation, the cells were exposed to blue, green, or red light once for 10 or 20 min or twice for 20 min per exposure with an interval of 1 h between exposures to avoid an increase in temperature, followed by further incubation up to 72 h. The controls were handled in the same manner except for the exposure. Cells were colorimetrically counted using a Cell Counting Kit (Dojin, Kumamoto) at several time-points. Specifically, 10 $\mu$l of a 5 m*M* solution of WST-1 (a colorimetric reagent) dissolved in 0.2 m*M* 1-methoxy PMS was added to each well, and light absorbance at 405 nm was measured 2 h later.

**Effects on cell death** Cell suspensions (100 $\mu$l) containing $3\times10^4$ to $1\times10^5$ cells/ml were inoculated into 96-well microplates (Costar 3917, Corning, Inc., Corning, NY). At 24 h after inoculation, the cells were exposed to blue light for 20 min, followed by further incubation up to 120 h. Dead and total cells were counted at several time-points by fluorometric assay based on the cell lysis and staining method (Cytotoxic Fluorotest, Wako, Osaka). Specifically, the intensity of fluorescence was measured using a fluorescence absorption multi-plate reader (Spectrafluor, Wako) at an excitation wavelength of 420 nm and an emission wavelength of 460 nm.

**Effects on colony formation** A cell suspension (5 ml) containing $1\times10^2$ cells/ml was inoculated into 6-cm-diameter dishes (Corning 430166, Corning, Inc.), and incubated for 24 h, then the cells were exposed to blue, green, or red light for 20 min. Each culture was incubated for 11 days without changing the medium. The culture was then washed once with 5 ml of cell-incubation medium, dried in air for 2 h, fixed with 5 ml of 70% ethanol for 10 min, dried in air for 24 h, stained with Giemsa's solution for 30 min, and dried in air again. The shape, number, and area of colonies and the cell count per colony were determined using an image analysis program (Win Roof 3.1, Mitani Corp., Kanazawa). Comparison of the effects of blue light and UVC was performed with or without a plastic cover during exposure to blue light or UVC for 20 min.

**Cell cycle analysis by flow cytometry** Cell suspensions (15 ml) containing $3\times10^3$ to $1\times10^5$ cells/ml were inoculated into incubation dishes (Omnitray 165218, Nunc, Inc., Naperville, IL) and incubated in FBS-free medium for 24 h. After the medium was changed to medium containing FBS, the cells were incubated for an additional 24 h. The cells were then exposed to blue light once for 20 min or twice for 20 min with an interval of 1 h between exposures. After exposure, the cells were incubated for 24, 48, 72, or 96 h, stained with propidium iodide (Sigma Aldrich, St. Louis, MO), and then subjected to flow-cytometric analysis of the cell cycle using a FACScan (Becton Dickinson) with CellQuest and ModFitLT 2.0 software (Becton Dickinson).

Analysis of the S phase of the cell cycle using 5-bromo-2′-deoxyuridine (BrdU, Sigma Aldrich) staining was carried out as follows. Cell suspensions (15 ml) containing $5\times10^3$ to $3\times10^4$ cells/ml were incubated in FBS-free medium for 24 h. The cells were then incubated in medium containing FBS for an additional 24 h. After incubation, the cells were exposed to blue light for 20 min. At 96 h after exposure, BrdU was incorporated for 30 min at a concentration of 10 $\mu M$. After the cells were collected, they were stained with fluorescein isocyanate (FITC)-conjugated anti-BrdU antibody (Becton Dickinson) and propidium iodide (Sigma Aldrich). Two-dimensional analysis of DNA content and BrdU incorporation was then carried out using the FACScan with CellQuest software.

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

## RESULTS

**Growth inhibition of B16 melanoma cells** When B16 melanoma cells were exposed to blue, green, or red light, cell growth was inhibited only by blue light in proportion to the exposure time (Fig. 2). The temperature change during exposure was negligible.

With regard to the time-course after exposure, in controls, the cells with an initial density of $1\times10^4$ or $3\times10^4$ cells/ml continued to grow up to 72 h. When the initial

cell density was $1\times10^5$ cells/ml, cell growth began to plateau after 24 h. In the blue light group, significant suppression of cell growth was observed from 24 h after exposure. The difference in cell growth between the exposed and control groups increased over time up to 72 h after exposure, with the percentages relative to controls at 72 h after exposure determined to be 30% and 33% for initial cell counts of $1\times10^4$ and $3\times10^4$ cells/ml, respectively (Fig. 3).

**Effects on cell death** In the control group, the increase in total cells (1799% of the pre-exposure level) was accompanied by an increase in dead cells (275%) after 120 h of incubation when the number of initially inoculated cells was $3\times10^4$ cells/ml. In the blue light group, neither total (101%) nor dead (29%) cells increased, indicating that inhibition of cell growth by exposure to blue light was not due to a loss of cell viability (Fig. 4). The number of dead cells in the blue light group was also significantly less than the number in the control group when the initial cell number was $1\times10^5$ cells/ml.

**Effects on colony formation** None of the lights, including blue light, showed a marked effect on the number of colonies that had formed at 11 days after exposure compared with the control group, with 137 colonies observed in the blue light group and 146 in the control group. However, the sizes of individual colonies were significantly reduced in the blue light group as compared with the control group and the red or green light groups, and the cell counts per colony were significantly smaller in the blue light group (Fig. 5 and Table I). Among the control, red, and green light groups, little difference was seen in cell morphology, and small cells with short, roundish, wedge-like forms were observed at a high density. On the other hand, in the blue light group, long, large, striated cells were seen at relatively low density. Even when the exposure intensities were adjusted to similar levels (2.27 mW/cm$^2$ for red, 2.26 mW/cm$^2$ for green, and 2.24 mW/cm$^2$ for blue), only blue light showed an inhibitory effect on colony growth.

Although exposure to UVC (wavelength 253.7 nm) for 20 min completely inhibited colony formation, this inhibitory effect was prevented by covering the dish with a plastic plate. On the other hand, the effect of blue light on colony formation was the same with or without a plastic cover (Fig. 6).

**Cell cycle analysis by flow cytometry** No apparent effects on the cell cycle of B16 melanoma cells were observed at 24 or 48 h after exposure to blue light, regardless of the exposure time. However, the percentage of G0/

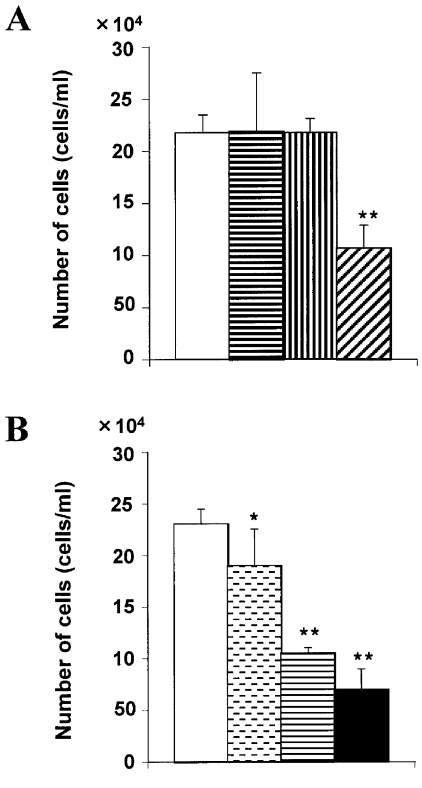


Fig. 2. The number of cells 48 h after exposure to red, green or blue light for 20 min (A) and to blue light once for 10 or 20 min or twice for 20 min (B). Cell suspensions (100 $\mu$l) of B16 melanoma containing $10^4$ cells/ml were inoculated into 96-well microplates. At 24 h after inoculation, the cells were exposed to light. A: □ control, ▤ red, ▥ green, ▨ blue. B: □ control, ▩ 10 min, ▤ 20 min, ■ 20 min×2. Data are expressed as mean±SD ($n$=8). * $P$<0.05, ** $P$<0.01 vs. control.

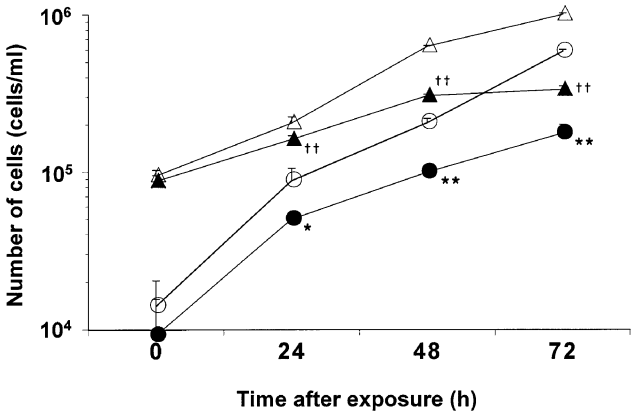


Fig. 3. Time-course of the growth of B16 melanoma cells after exposure to blue light for 20 min at 24 h after inoculation. Initial cell densities were $1\times10^4$ cells/ml for control 1 and blue 1 and $3\times10^4$ cells/ml for control 2 and blue 2. ○ control 1, ● blue 1, △ control 2, ▲ blue 2. Data are expressed as mean±SD ($n$=4–5). * $P$<0.05, ** $P$<0.01 vs. control 1; †† $P$<0.01 vs. control 2.

G1 phase cells was increased and that of S phase cells was decreased at 72 h after exposure to blue light for 20 min (Fig. 7, Table II). These changes were more pronounced in cells that were exposed twice, accompanied by an increase in the percentage of G2/M phase cells. For single exposure, this was also the case after 96 h. Additionally, marked inhibition of the S phase was detected by two-dimensional analysis of DNA content and BrdU incorporation at 96 h after exposure (Fig. 8).

## DISCUSSION

The results of the present study indicate that blue light, one of the three primary colors composing the visible spectrum, selectively inhibits the growth of B16 melanoma cells. The inhibitory effect was enhanced as the exposure time was increased. No increase in the number of dead cells was seen at 72 or 120 h after exposure to blue light. The finding that the decrease in colony number was slight but the size of colonies and number of cells per colony were markedly reduced suggests that the inhibition of cell growth was due not to cell death but to inhibition elsewhere in the cell cycle. Interestingly, these results confirm the previous finding that the inhibitory effect of exposure to blue light continues to be seen even when cells are passaged for three generations (unpublished data). The shapes of the cells in the colonies were markedly changed by exposure to blue light, suggesting a functional alteration in the cells. The detailed molecular mechanism,

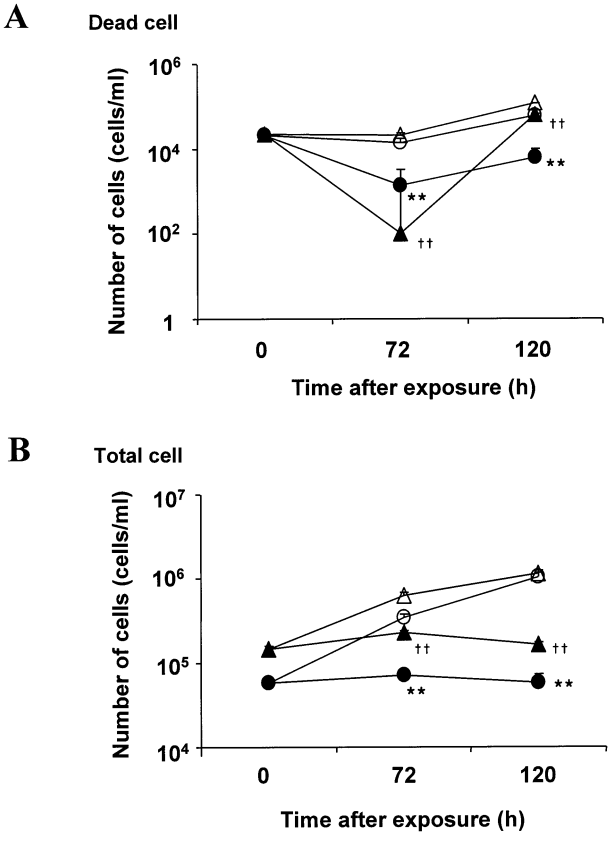


Fig. 4. Time-course of numbers of dead (A) and total (B) B16 melanoma cells after exposure to blue light for 20 min. Initial cell densities were $3\times10^4$ cells/ml for control 1 and blue 1; $1\times10^5$ cells/ml for control 2 and blue 2. Data are expressed as mean±SD ($n$=6). ** $P$<0.01 vs. control 1, †† $P$<0.01 vs. control 2. ○ control 1, ● blue 1, △ control 2, ▲ blue 2.

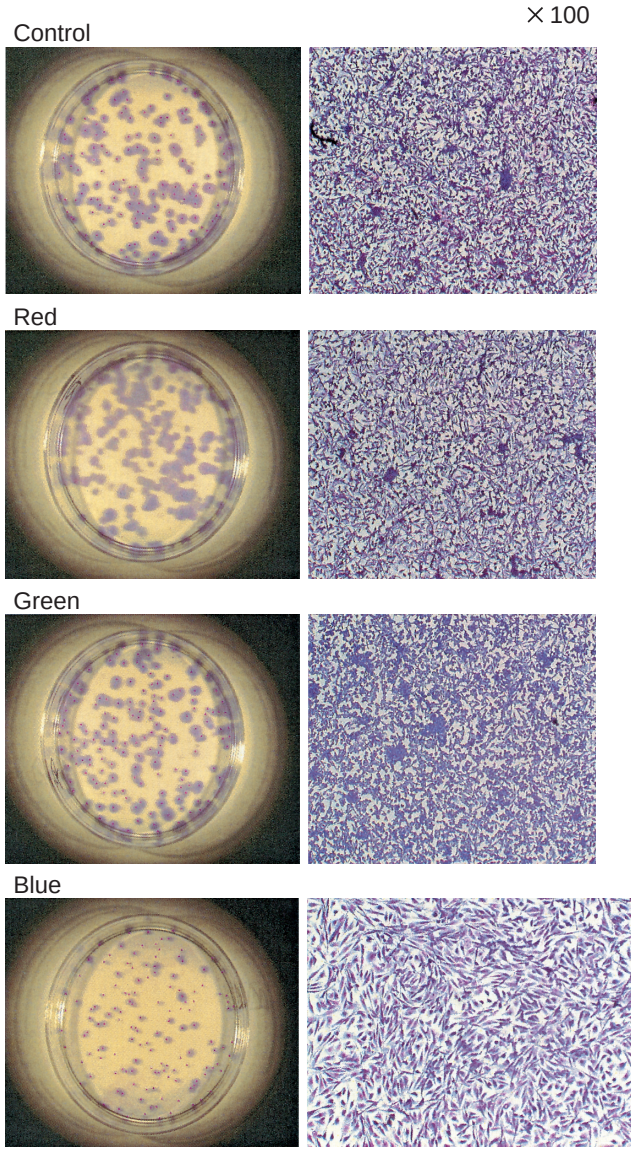


Fig. 5. Colonies formed and microscopic appearance 11 days after exposure to red, green, or blue light for 20 min.

including whether RNA synthesis and protein synthesis are also altered in the cells, remains to be elucidated. The difference between the effects of UVC, which were prevented by covering the dish with a plastic plate, and blue light, which were not affected by a plastic cover, also suggests that the mechanism of action differs between UVC and blue light emitted by LEDs. We also found that the inhibitory effects on colony formation of UVA (wavelength 320–380 nm) were prevented by covering the dish (unpublished data).

In the present study, apparent increases in the G0/G1 and G2/M phases and a decrease in the S phase were confirmed at 72 h after exposure to blue light by analyzing the amount of DNA by flow cytometry. These results suggest that the inhibitory effect of blue light on the growth of B16 melanoma cells may result from an effect on the cell cycle, specifically, inhibition of progression from the G1 to S phase and prolongation of the M phase. In addition, the finding that the number of cells in the S phase was decreased at 96 h after exposure based on analysis of the S phase using BrdU also suggests inhibition of the transition from the G1 to S phase. UVB irradiation has been reported to arrest the cell cycle at the G1 and S phases in rat keratinocytes[23] and UVA or UVB irradiation was found to cause a temporary accumulation of S phase cells in V79 Chinese hamster lung fibroblasts and 3T3 Swiss albino mouse fibroblasts.[24] However, the results of the present study involving blue light are clearly different from those of earlier reports because blue light induced an increase in the G1 phase and a decrease in the S phase 72 h or more after exposure.

Table I. Number, Area, and Cell Counts of Colonies Formed 11 Days after Light Exposure

| | Number of colonies | Area of colonies (%) | Cell counts per colony |
|---|---|---|---|
| Control | 146±3 | 35.4±4.8 | 30 281±3 846 |
| Red | 152±8 | 35.9±5.5 | 28 728±3 643 |
| Green | 150±8 | 33.8±3.1 | 26 313±3 231 |
| Blue | 137±4** | 8.1±1.5** | 5 363±1 311** |

Data are expressed as mean±SD ($n$=8). ** $P$<0.01 vs. control.

We previously confirmed that inhibition of the growth of HL-60 cells by exposure to blue light is partially released by the reactive oxygen species scavenger catalase. However, a biomarker of oxidative injury of DNA, 8-hydroxydeoxyguanosine (8-OHdG), was not increased until 48 h after exposure to blue light for 20 min (unpublished results), suggesting that brief exposure to blue light

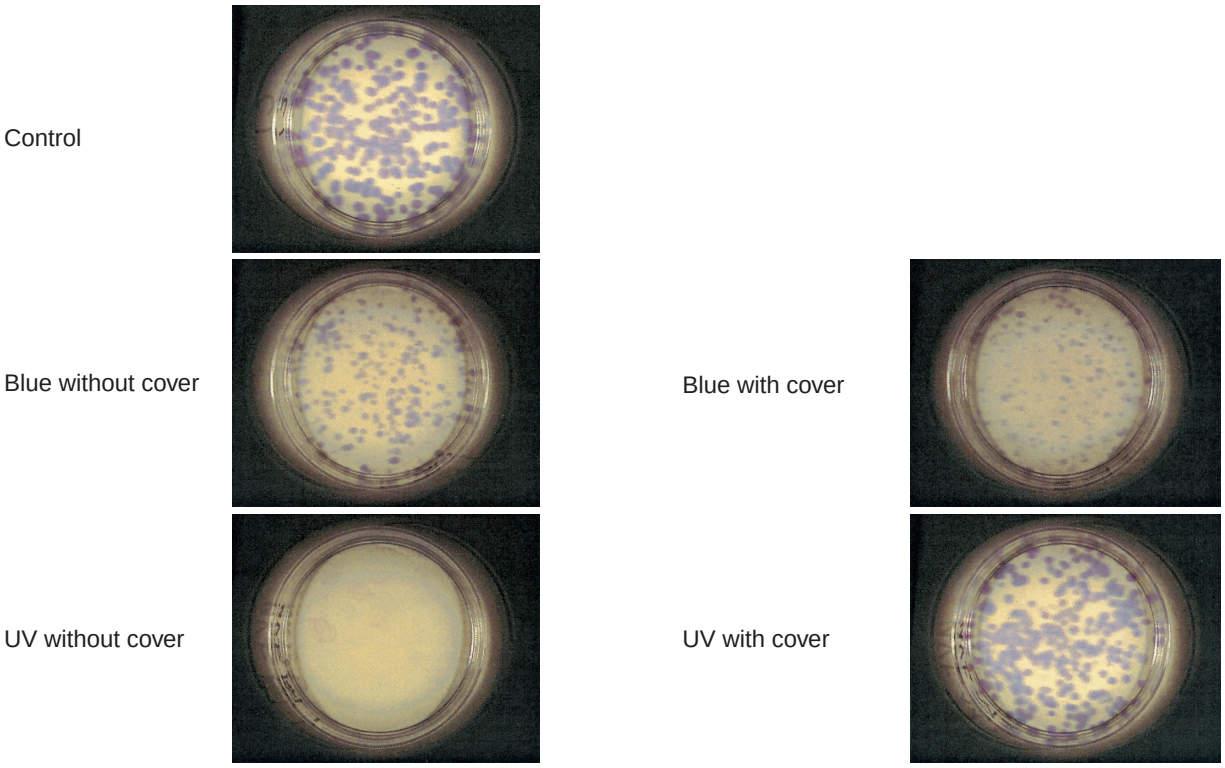


Fig. 6. Colonies formed 11 days after exposure to blue light or UVC for 20 min with or without a plastic cover.

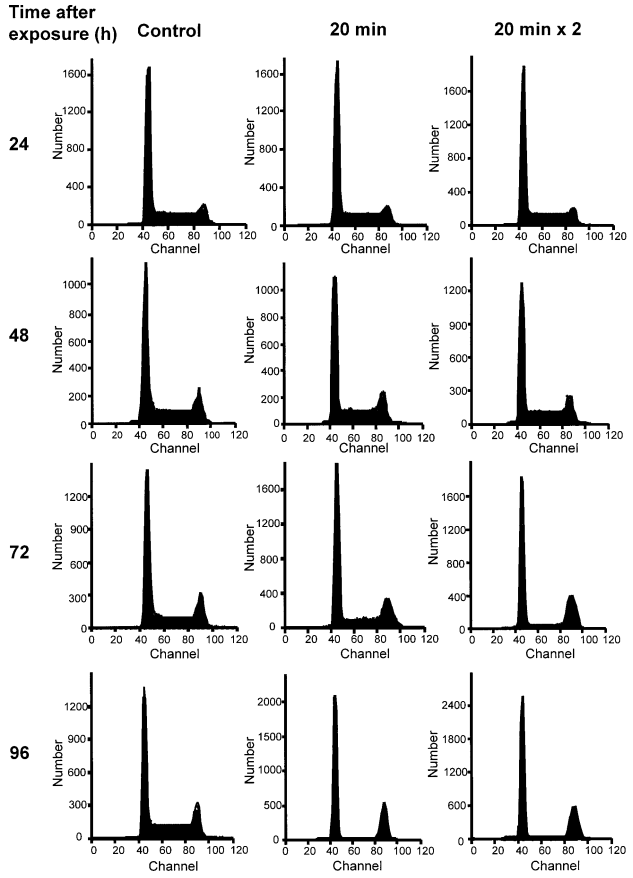


Fig. 7. Flow-cytometric analysis of B16 melanoma cells after exposure to blue light for one or two 20-min periods.

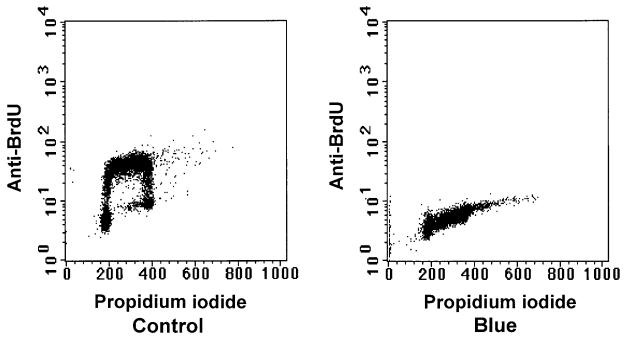


Fig. 8. Two-dimensional flow-cytometric analysis of DNA content and BrdU incorporation at 96 h after exposure to blue light for 20 min.

may affect factors related to the growth of B16 melanoma cells. It is necessary to examine whether the levels of p53 and related proteins are altered in the cells exposed to blue light, and whether apoptosis or cell death may be induced long after exposure to blue light.

The possibility that toxic products may be formed in the media by the action of blue light was considered, but in this study little effect was seen when we irradiated the medium first and then added the cells, and blue light also inhibited cell growth in phosphate-buffered saline solution (unpublished data).

Although the inhibition of cell growth by exposure to blue light is expected to be related to inhibition of DNA synthesis and cell-division processes, the detailed molecu-

Table II. Data from Flow-cytometric Analysis of B16 Melanoma Cells Exposed to Blue Light

| Group | G0/G1 | S | G2/M |
|---|---|---|---|
| 24 h after exposure (%, $n$=5) | | | |
| Control | 52.85±2.55 | 37.60±3.13 | 9.55±2.04 |
| Blue 20 min×1 | 53.24±2.63 | 37.13±2.30 | 9.63±1.45 |
| Blue 20 min×2 | 53.94±5.40 | 36.34±4.06 | 9.73±2.28 |
| 48 h after exposure (%, $n$=6) | | | |
| Control | 44.69±3.28 | 42.02±2.44 | 13.30±1.21 |
| Blue 20 min×1 | 44.87±1.85 | 42.36±1.38 | 12.77±1.15 |
| Blue 20 min×2 | 47.17±4.31 | 39.60±5.51 | 13.23±1.76 |
| 72 h after exposure (%, $n$=6) | | | |
| Control | 45.06±4.70 | 43.18±7.30 | 11.75±3.03 |
| Blue 20 min×1 | 54.44±7.00* | 32.73±5.96* | 12.83±3.18 |
| Blue 20 min×2 | 69.29±5.32** | 9.85±4.99** | 20.86±1.26* |
| 96 h after exposure (%, $n$=6) | | | |
| Control | 42.92±2.21 | 43.60±2.54 | 13.49±1.06 |
| Blue 20 min×1 | 70.85±1.79** | 4.09±2.06** | 25.06±2.53** |
| Blue 20 min×2 | 81.22±1.66** | 1.85±1.09** | 16.94±2.34* |

Data are expressed as mean±SD. * $P$<0.05, ** $P$<0.01 vs. control.

lar mechanism of this action, including possible formation of photoproducts and analysis of expression of cell cycle proteins, remains to be elucidated. We are currently studying the effectiveness of blue light in various human tumor cells such as A357, ACHN, HeLa S3, Daudi, K562, MRC7, PC12, Raji and MRC5, and have observed significant growth inhibition (unpublished results). However, with regard to melanocytes (human non-malignant cells), growth inhibition was not obtained, possibly because the exposure and observation times were short (unpublished results). Longer exposure and observation times should be examined. Recently, we found in an experiment with mice that when HeLa S3 cells were implanted subcutaneously or beneath the renal membrane, subsequent exposure to blue light suppressed the growth of the implants (unpublished data). We also found that in rats with erythroblastic leukemia induced by 1-ethyl-1-nitrosourea, exposure of the peripheral blood to blue light resulted in a decrease in the number of leukemia cells, without any change in number of the normal lymphocytes (unpublished data). Such studies are expected to provide a better understanding of the effects of visible lights on cell proliferation, and may offer new approaches to the treatment of cancer in humans. The new LEDs that have recently become available show promise as useful tools for studying the effects of each component of the visible spectrum on the living body.

**ACKNOWLEDGMENTS**

The authors would like to thank Mr. Y. Shimizu of Nichia Corp. for constructing the LED apparatus and Mr. T. Fujikura for his technical assistance.

*(Received October 26, 2001/Revised February 22, 2002/ Accepted February 28, 2002)*

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