Augmentation of the inhibitory effect of blue light on the growth of B16 melanoma cells by riboflavin

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Abstract. We have demonstrated that blue light has anticancer effects in cultured cancer cells and tumor-bearing animals. Based on our experimental findings, in addition to cytostatic activity that suppresses the proliferation of B16 melanoma cells, blue light may exert cytocidal activity through interaction with vitamin(s) contained in the culture medium. The present study was undertaken to identify the specific vitamins with which blue light interacts and to investigate the factors responsible for its cytocidal activity. B16 melanoma cells were incubated in media supplemented with various vitamins and exposed to blue light for 10 min. Cell necrosis was observed only in media containing riboflavin (0.4 mg/l). The effects of other components of visible light on riboflavin were also studied. Riboflavin-containing media were exposed to light of each of the three primary colors (red, green and blue) and the effects on the colony-forming capacity of B16 melanoma cells were evaluated. Cell necrosis was induced only in media exposed to blue light. The effects of riboflavin increased in a concentration-dependent manner in the range from 0.3 to 1.0 mg/l in blue-light-exposed media and were antagonized by the presence of catalase (200 U/ml). These findings suggest that cell necrosis is probably induced by active oxygen species such as hydrogen peroxide formed by the reaction of riboflavin with blue light.

Introduction

Although numerous studies have been carried out to investigate the relationships between light and disease, with special attention paid to the application of radiation and ultraviolet rays (UV) to cancer treatment (1-5) and the evaluation of carcinogenic (6-10) and immunosuppressive (11) effects, the effects of visible light are still not well understood in the medical field. We examined the effects of components of visible light on the growth of cancer cells using light-emitting diodes (LEDs), which have selective light-emission spectra, and found that blue light suppressed the growth of B16 melanoma cells in a time-dependent manner (12). The effects of blue light appeared to be attributable to the inhibition of DNA synthesis and cell division. When the blood of rats with 1-ethyl-1-nitrosourea (ENU)-induced leukemia was exposed to blue light for 3 h during extracorporeal circulation, the growth of leukemic cells was suppressed, with no significant difference in the growth of normal lymphocytes, compared to the non-exposure control group (13). When B16 melanoma cells exposed to blue light and incubated for 7 days were injected intravenously into mice, the metastasis of B16 melanoma cells to the lung was suppressed (14). The induction of skin tumors by 12-O-tetradecanoylphorbol-13-acetate in the v-Ha-ras transgenic mouse was also suppressed by blue light exposure (15). An interesting phenomenon was noted in the course of these studies: when the culture medium for the B16 melanoma cells was switched from Eagle's MEM (Nihon Pharmaceutical Co., Ltd., Tokyo, Japan; hereinafter referred to simply as 'MEM') to Dulbecco's MEM (D5796, Sigma Aldrich, St. Louis, MO; hereinafter referred to as ‘D-MEM’), the effects of exposure to blue light were markedly increased, and powerful cytocidal activity, which was not seen in the experiments using MEM, was observed. Preliminary investigations revealed that some vitamin(s) contained in D-MEM might be related to these effects (unpublished data). We therefore conducted additional studies in order to identify the vitamin(s) reacting with the blue light as well as the cytocidal factor(s) formed in the cell culture medium following exposure to blue light.

Materials and methods

LED device. The apparatus was constructed using red, green and blue LEDs (Nichia Corp., Tokushima, Japan) as reported previously (12). Each LED was driven by direct current from a standard power supply (S82K-10024, Omron, Tokyo, Japan). The blue LEDs used for evaluation of the effects of exposure to blue light had the following specifications: current 30.1 mA, wavelength 470 nm and irradiance 5.7 mW/cm². The LEDs used for comparison of the effects of light of the three primary colors had the following specifications: red = current 32.0 mA, wavelength 634 nm and irradiance 2.27 mW/cm²; green = 30.5 mA, 518 nm and 2.26 mW/cm²; and blue = 8.0 mA, 470 nm and 2.24 mW/cm².

Cell culture. B16 melanoma cells (JCRB0202, Human Science Research Resources Bank, Osaka, Japan) cultured in MEM
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% CO₂/95% air atmosphere.

Culture media. Eagle's MEM (MEM) was purchased from Nihon Pharmaceutical Co., Ltd. (Tokyo, Japan). Dulbecco's MEM (D5796, D-MEM) was purchased from Sigma Aldrich (St. Louis, MO).

Effects of culture medium on the growth of B16 melanoma cells. A cell suspension (2 ml) in MEM or D-MEM (supplemented with 10% FBS) containing 5x10⁵ cells/ml was inoculated into 6-well microplates (Becton Dickinson, Franklin Lakes, NJ). After incubation for 24 h, the cultures were exposed to blue light for 20 min and then incubated for an additional 4 days. Another set of 6-well microplates containing MEM or D-MEM (2 ml/well) was exposed to blue light for 20 min. Immediately after blue light exposure, each medium was used to replace 1.8 ml of the supernatant of the cell suspensions (2 ml) in MEM or D-MEM (supplemented with 10% FBS) containing 5x10⁵ cells/ml. The cell cultures were then incubated for 4 days. The number of viable cells was then counted by trypan blue staining.

Culture medium with vitamins. MEM was supplemented with 10% FBS and amino acids (MEM amino acid solution, Life Technologies, Inc.) to adjust the concentration of each amino acid to closely match that of D-MEM, the amino acid concentration of which is about twice that of MEM. The resultant solution was then supplemented with the following vitamins so as to obtain final vitamin concentrations equal to those in D-MEM: choline chloride (4 mg/l, Sigma Aldrich), folic acid (4 mg/l, Takeda, Osaka, Japan), myo-inositol (7.2 mg/l, Sigma Aldrich), niacinamide (4 mg/l, Nicpon Roche, Tokyo, Japan), calcium pantothenate (4 mg/l, Sigma Aldrich), pyridoxine hydrochloride (4 mg/l, Sigma Aldrich), riboflavin (0.4 mg/l, Sigma Aldrich) and thiamin HCl (4 mg/l, Nicpon Roche).

Death of B16 melanoma cells exposed to blue light in medium containing various vitamins. Cell suspensions (2 ml) suspended in MEM supplemented with 10% FBS containing 1x10⁵ B16 melanoma cells/ml were inoculated into 6-well microplates. At 24 h after inoculation, other 6-well microplates containing 2 ml/well of each test solution (prepared as described in the previous section) were exposed to blue light for 10 min. Immediately after light exposure, each test solution was used to replace 1.8 ml of the supernatant of the cell cultures. The cell cultures were incubated for an additional 48 h. The cultures were then washed once with 5 ml of Dulbecco's phosphate-buffered saline (PBS, Sigma Aldrich), 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 solution for 30 min and dried in air again. Photographs were taken of the colonies that had formed in each dish. In the control group, the supernatant of the cell cultures was replaced with 1.8 ml of MEM supplemented with 10% FBS and amino acids but not vitamins, followed by the same procedures.

Wavelength dependency of suppressive effects of riboflavin on colony formation. Riboflavin was added to MEM supplemented with 10% FBS to a final concentration of 0.4 mg/l. A 5-ml suspension of B16 melanoma cells (5x10⁵ cells/ml) in MEM supplemented with 10% FBS was inoculated into dishes (6 cm in diameter) and incubated for 24 h at 37°C in a 5% CO₂/95% air atmosphere. Other dishes containing 5 ml of the riboflavin-supplemented medium were exposed to red, green or blue light for 20 min. Control dishes were not exposed to light. Immediately after light exposure, the riboflavin-supplemented medium was used to replace 4.5 ml of the supernatant of the cell cultures. The cell cultures were then incubated for 11 days. After incubation, the cells in each dish were fixed with 70% ethanol and stained with Giemsa solution. Photographs were taken of the colonies that had formed in each dish.

Effects of catalase on suppression of growth of B16 melanoma cells by riboflavin and blue light. The basic medium, composed of MEM and 10% FBS, was supplemented with riboflavin at varying concentrations (0.1, 0.3, 1.0 and 3.0 mg/l). A 100-µl suspension of B16 melanoma cells (10^4 cells/ml) in MEM supplemented with 10% FBS was inoculated into 96-well microplates (3075, Becton Dickinson) and incubated for 24 h at 37°C in a 5% CO₂/95% air atmosphere. Ten microliters of catalase (200 U/ml, Wako, Osaka, Japan) or 10 µl of MEM supplemented with 10% FBS was added to other microplates containing 100 µl of the riboflavin-supplemented medium, and the plates were then arranged on a clean bench and exposed to blue light for 7 min. Immediately after light exposure, the medium was used to replace 80 µl of the supernatant of the cell cultures. In the control group, the same procedures were performed except that the plates containing the test solutions were not exposed to blue light. The cell cultures were then incubated for 48 h. During the experiments, the microplates were shielded with aluminum foil at all times except during the period of exposure to blue light. Cells were colorimetrically counted using a Cell Counting Kit (Dojin, Kumamoto, Japan). Specifically, 10 µl of a 5 mM solution of WST-1 (a colorimetric reagent) dissolved in 0.2 mM 1-methoxy PMS was added to each well and light absorbance at 405 nm was measured 2 h later.

Statistical analysis. The results are expressed as mean ± SD. For comparisons 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 t-test was used. For comparisons among three or more groups, Dunnett’s test was employed.

Results

Effects of culture medium on the growth of B16 melanoma cells. For incubation in MEM, exposure of both the cells and medium to blue light resulted in marked suppression of cell growth after 4 days, while exposure of the medium alone to blue light did not result in any significant suppression of cell growth. For incubation in D-MEM, either exposure of both the medium and cells to blue light or exposure of the medium alone to blue light resulted in complete loss of cell viability, with no cells adherent to the bottom of the dishes (Fig. 1).
Death of B16 melanoma cells exposed to blue light in medium containing various vitamins. The proportion of cells adherent to the bottom of the dish did not differ markedly among the group that was not exposed to blue light, the group exposed to blue light in medium without vitamin supplementation and the group exposed to blue light in medium supplemented with vitamins other than riboflavin. However, the cells incubated in riboflavin-supplemented medium and exposed to blue light showed complete loss of viability, and no cells were found adherent to the bottom of the dishes (Fig. 2).

Light wavelength dependency of the suppressive effect of riboflavin on colony formation. Colony formation, evaluated in terms of the number of colonies, the size of the colonies and the percent area of the bottom of the dish covered by colonies, did not differ significantly between the non-blue-light-exposed
cells (10⁴ cells/ml) was inoculated into 96-well microplates containing the test solutions were used to replace 80% of the cell culture. Immediately after blue light exposure, the plates were incubated for 5 min after catalase was added (200 U/ml) to each well, the plates were incubated for 48 h. Another set of microplates were filled with 100 µl of a suspension of B16 melanoma cells (5x10⁴ cells/ml) was inoculated into dishes (6 cm in diameter) containing the riboflavin-supplemented basic medium, and the cells were incubated for 24 h. Another set of dishes inoculated with 5 ml of the cell suspension were exposed to red, green or blue light for 20 min. After incubation for 11 days, the cells in each dish were stained with Giemsa solution.

Effects of catalase on suppression of B16 melanoma cell growth by riboflavin and blue light. When the supernatant of the cell cultures was simply replaced with MEM supplemented with riboflavin (0.1, 0.3, 1.0 or 3.0 mg/l) or with catalase, the growth of B16 melanoma cells was not affected. When the supernatant was replaced with medium with a riboflavin concentration of 0.1 mg/l (equivalent to the riboflavin concentration in MEM) with exposure to blue light for 7 min, the results did not differ from those for the non-blue-light-exposed group after incubation for 48 h. However, in the 0.3, 1.0 and 3.0 mg/l riboflavin-supplemented groups, cell growth was significantly suppressed, with reductions in cell counts to 61.4, 4.1 and 9.3% respectively, relative to the 0.1 mg/l riboflavin-supplemented group without exposure to blue light (Fig. 4). Microscopic examination showed that most of the cells cultured in media supplemented with riboflavin at 1.0 or 3.0 mg/l were necrotic and had detached from the bottom of the microplates. The addition of catalase (200 U/ml) resulted in significant recovery of the reduction in cell counts induced by blue light in the riboflavin-supplemented media (0.3, 1.0 and 3.0 mg/l), with cell counts 89.1, 91.1 and 74.8% that of the 0.1 mg/l riboflavin-supplemented group without exposure to blue light (Fig. 4). Microscopic examination also confirmed a marked reduction in cell death in the catalase-treated group.

Discussion

In the present study, riboflavin was identified as an important factor involved in the necrosis of B16 melanoma cells incubated in D-MEM and exposed to blue light. Cell necrosis was induced even when the medium alone was exposed to blue light before the cells were inoculated, and this effect became more pronounced as the concentration of riboflavin in the medium was increased in the range from 0.3 to 1.0 mg/l. Although the calculated number of cells was greater in medium with a riboflavin concentration of 3.0 mg/l than in a medium with a riboflavin concentration of 1.0 mg/l, this is probably attributable to the yellow color of concentrated riboflavin, which may have affected the absorbance used for counting the cells. Microscopic examination showed that most cells were necrotic and had detached from the bottom of the microplates at both riboflavin concentrations. Of the three primary colors of visible light, only blue light induced cell necrosis when the cells were incubated in riboflavin-supplemented medium.

When the concentration of riboflavin in MEM was low (0.1 mg/l), the growth of B16 melanoma cells did not appear to be affected by prior exposure of the medium alone to blue light. At low riboflavin concentrations, exposure of the cells to blue light alone was required for the suppression of cell growth. The suppression of cell growth at such low riboflavin concentrations was caused by suppressed cell cycling, but not by cell death (12). When the medium contained high concentrations of riboflavin (>0.3 mg/l), cell death was induced, and this effect was significantly suppressed by the presence of catalase. Since catalase is known to be a hydrogen peroxide scavenger, this latter finding suggests that the factor responsible for cell death in the presence of high concentrations of riboflavin is probably hydrogen peroxide or a similar active oxygen species. In cultures of B16 melanoma cells in MEM...
containing a low concentration of riboflavin (0.1 mg/l), the suppression of cell growth induced by exposure to blue light was not affected by treatment with catalase (unpublished data). This suggests the involvement of factors other than active oxygen species in the suppression of B16 melanoma cell growth by blue light, in the presence of a low concentration of riboflavin.

It was previously reported that the reaction of riboflavin with UVA yields oxygen radicals such as singlet oxygen, superoxide anions, OH radicals and hydrogen peroxide (16,17). We also confirmed that necrosis of B16 melanoma cells occurs when the cells are incubated in D-MEM exposed in advance to UVA or UVC, without direct exposure of the cells to such light (unpublished data). Recently, studies have been conducted to investigate the possibility of employing oxygen radicals (formed by the reaction of UVA with riboflavin) in the treatment of blue nevus (18) and erosive corneal processes (19). The combination of blue light and photoreactive substances (riboflavin etc.) is also expected to be useful for the treatment of various conditions such as cancers, stains, blotches and erosive corneal processes.

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References