Blue Light is Phototoxic for B16F10 Murine Melanoma and Bovine Endothelial Cell Lines by Direct Cytocidal Effect

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Abstract. *The large number of studies devoted to the effect of ultraviolet light on biological systems, contrasts with the lack of experimental data concerning the direct effects of visible light. It has been shown that blue light inhibited the growth of B16F10 melanoma cell lines and reduced the percentage of S phase cells. Yet these effects are poorly understood. Materials and Methods: Two cell lines and irradiation with blue light were used. Cell mortality and a possible mechanism of action were investigated. Results: Exposure of B16F10 melanoma and bovine endothelial cells to blue light (wavelength 450 nm, 10 J/cm^2 from a Waldman lamp) induced a rapid and large reduction in viability followed by the death of virtually all the irradiated cells within 24 h. These results led us to expose a patient with haemorrhagic cutaneous melanoma metastasis to blue light. Irradiation led to an immediate arrest of haemorrhage, an inhibition of tumour growth and extensive tumour necrosis 24h after irradiation. Conclusion: Exposure to blue light may offer new approaches to the treatment of superficial skin carcinomas in humans.*

Melanoma is highly resistant to conventional chemotherapeutic agents and novel approaches are needed especially to treat subcutaneous tumours. Photodynamic therapy using cancer-designed photosensitizers and laser beams has been increasingly used in clinical medicine. The visible light at a wavelength corresponding to the drug absorption activates these agents and induces highly cytotoxic products: singlet molecular oxygen and oxygen radicals (1). Most papers have used red and sometimes infrared light to penetrate deeper into tissues and these wavelengths have previously displayed significant efficacy in treating pigmented melanoma. Blue light has rarely been used in photodynamic therapy due to its poor penetration in the skin. The effects of blue light have mainly been used in humans to treat hyperbilirubinaemia in infantile jaundice (2). In a limited number of studies, blue light has been shown to disrupt mitosis and mitochondrial activity (3). Other studies have suggested that blue light exerts a cytostatic effect on B16 melanoma cells (4). Recently, Lockwood *et al*. have demonstrated that blue light induced the generation of reactive oxygen species (ROS) in epithelial cells (5). For this reason, blue light has been proposed for a treatment of various carcinomas including melanoma, haematopoietic cell line carcinomas, squamous cell carcinoma *etc*. The mechanisms by which blue light interacts with its biological targets and provokes cellular damage still remain unclear.

Abbreviations: DMEM: Dulbecco's modified Eagle's medium, FCS: fetal calf serum, EJG: bovine capillary endothelial cell line, ROS: reactive oxygen species, MTT: 3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, PBS: phosphate-buffered saline, TBARS: thiobarbituric acid reactive substance, MDA: malondialdehyde, LPO: lipid peroxydation.

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In this study, the effects of blue light on melanoma cells and endothelial cells in comparison to red light were examined. Also a patient with haemorrhagic subcutaneous metastasis of melanoma was exposed to blue light.

Materials and Methods

Cells. B16F10 melanoma cells were obtained from the Oncology Center (Dr Perron Toulouse, France). The cells were grown to confluence in DMEM (Dulbecco's Modified Eagle's Medium, Gibco BRL, France) containing 10% fetal calf serum (FCS) (BioWhittaker, Walskerville, MD, USA) without antibiotics and left undisturbed in a 37˚C, 5 % CO_2 incubator. The culture medium was changed every 3 days. The cells were subcultured by dispersal with trypsin-EDTA and replated at a 1:6 split.

A capillary endothelial cell line EJG was obtained from the American Type Culture Collection (ATCC, LGC Standards Sarl 6, rue Alfred Kastler BP 83076-67123 Molsheim Cedex, France) and was cultured in DMEM (Invitrogen, BP 9695613 Cergy Pontoise Cedex) supplemented with 10% FCS, 2 mM L-glutamine, 1% non-essential amino acids (Invitrogen) and antibiotics: 100 U/ml penicillin and 100 μg/ml streptomycin in a humidified 5% CO_2 atmosphere at 37˚C.

PDT 450L. WALDMANN. A mobile unit was equipped with a three module-reflecting head revolving around two axes. The PhotoDynamic Therapy *PDT 450 L* was fitted with 12 compact fluorescent lamps of the Waldmann F-36W/blue V type, 36W. The special arrangement of the lamps and special reflector geometry for optimal intensity created a homogenous irradiation field and thus uniform and intense irradiation. These lamps irradiated within the 380-470 nm range with a maximum at 420nm. The irradiation unit was ventilated by integrated fans.

PhotoDyn 501.COSMEDICO. The "well-being distance" is more than 25cm. At a distance of 25cm from the device (length of the spacing rod), the area of the homogeneous irradiation field had a diameter of 10 cm. The orange filter allows red light therapy with a spectrum from 600 nm to 1400nm with a maximum at 800 nm.

Irradiation protocol. Cells were seeded at 105 cells/ml in 96-well plates after trypsinization. After 48h, the cells were washed twice with PBS and incubated for 2h with medium in a humidified 5 % CO_2 atmosphere at 37˚C. No photosensitizer was added to the wells. The cells were then irradiated with the Waldman Lamp (blue light, at 450 nm) and the Cosmedico Lamp (red light, at 800nm) at room temperature (25˚C). The fluence rate was 10 W per cm^2. The exposure time was adjusted to obtain a 50J/cm^2 fluence (red light) and 10J/cm^2 or 20J/cm^2 (blue light). The distance between the end of the optical fiber and the microplates were set to 37 cm (blue light) and 25 cm (red Light). During cell illumination temperature was monitored at the top of the microplate.

Cytotoxic and photocytotoxic studies. Cell viability was measured by the MTT [3-(4.5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric assay. Optical density measurements were performed before and then immediately after the end of irradiation, and 24 h, 48 h, 72 h and 96 h after the end of the irradiation. At the time of counting, 50 μl of phosphate-buffered saline (PBS)-MTT solution (1.5 mg/ml PBS) were added and incubated for one h at 37˚C. One h later, DMSO was added to all the wells which were incubated for further 15 min at 37˚C according to the method described by Mosman *et al*. (6).

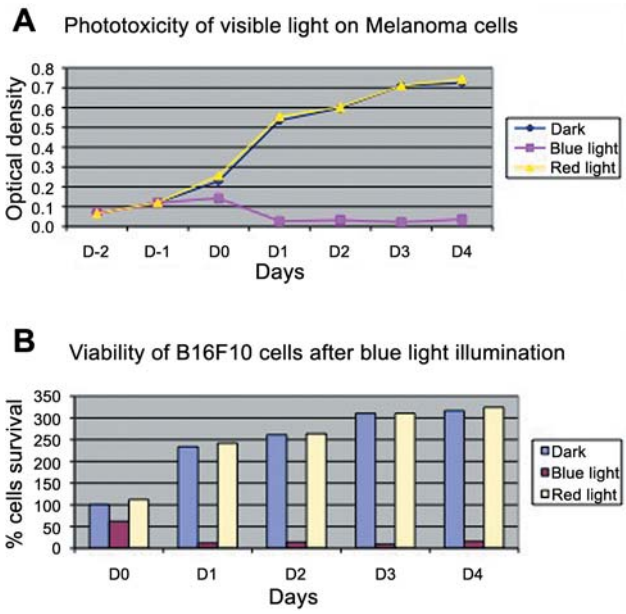

Figure 1. *Phototoxicity of red and blue light on murine melanoma B16F10 cells. 1A: Cell viability assesses by MTT assay at 570 nm. 1B: Percentage of viable cells after exposure to 50 J/cm^2 (red light) and 20j/cm^2 (blue light).*

The optical densities of the microplates were then read at 570 nm with an ELISA microplate reader (Multiskan® EX, Microplate photometer).

To determine the accuracy of the MTT assays for each experiment, two measurement points were routinely checked using the trypan blue exclusion test on a random basis.

Lipid peroxidation. Lipid peroxydation (LPO) in illuminated B16F10 cells was determined by measuring the formation of thiobarbituric acid reactive substance (TBARS). Lipid peroxidation products (malondialdehyde (MDA), other aldehydes and lipid hydroxiperoxides) reacts with thiobarbituric acid (TBA) to yield a complex (so called TBARS), spectroscopically detected at 532 nm. The reaction is favored under acidic conditions and at high temperature. The assay was conducted as follows: 1 ml of treated cells (5 millions/ml) was mixed with 500 μl of 10% Trichloracetic acid (TCA) containing 0.5% TBA. Samples were heated to 95˚C for 40 minutes. The reaction was stopped by cooling test tubes in ice. The colored complex was extracted by addition of 1 ml *n*-butanol, for which absorbance was measured at 532 nm on a Shimadzu (UV-2401PC) spectrophotometer. The concentration of TBARS formed was expressed as nmol of MDA/millions cell using MDA as standard.

Statistical analysis. Data are expressed as mean±SD. For comparison between the visible light (blue or red) exposure group and the control group, the Kruskal-Wallis test was used to detect differences in the average.

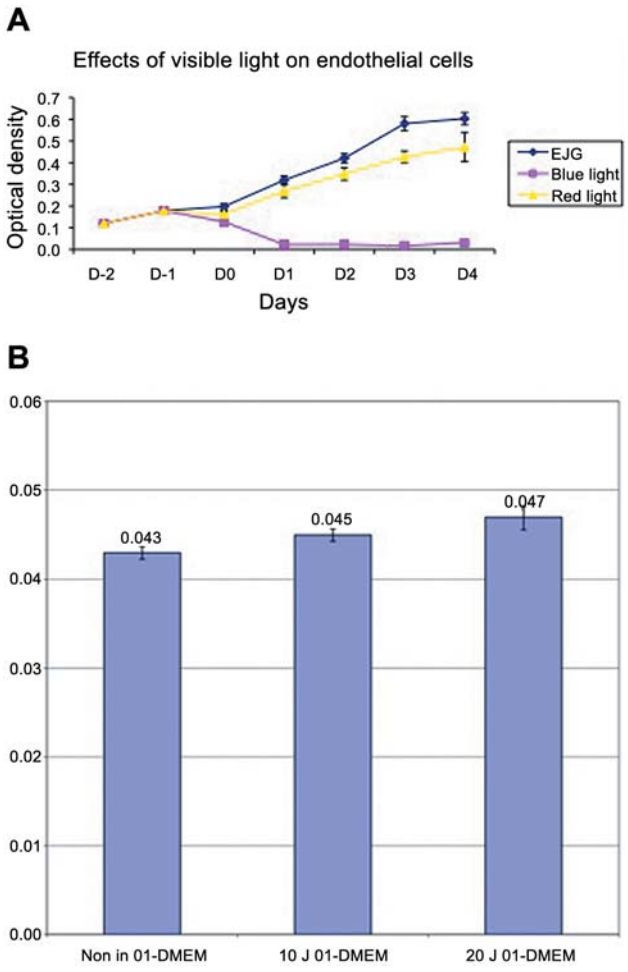

Figure 2. *A. Phototoxicity of blue and red light on bovine endothelial cells, by the colorimetric MTT assay. B. Lipid peroxidation in B16F10 cells illuminated by blue light with two fluences 10J/cm^2 or 20J/cm^2.*

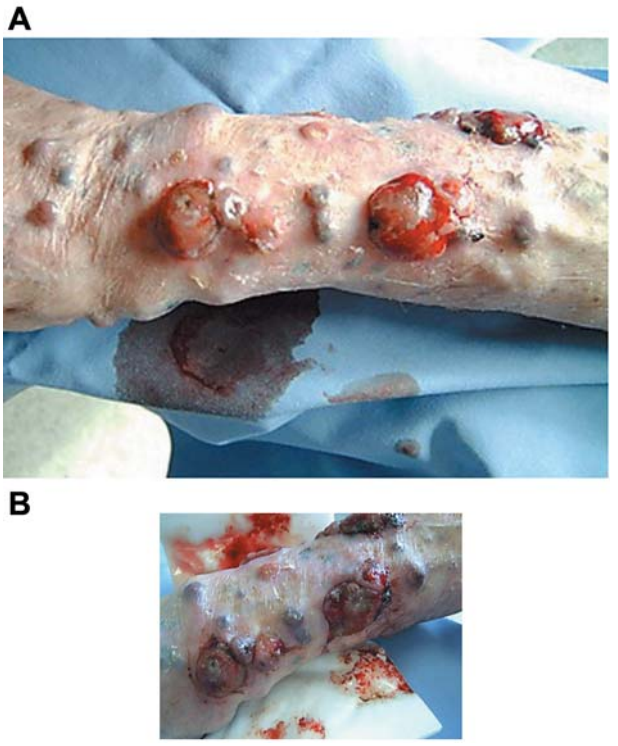

Figure 3. *A. Subcutaneous metastasis on the right forearm before irradiation. B. Clinical appearance after illumination without photosensitizer 5 consecutive days.*

Results

Visible light phototoxicity on B16F10 and EJG cells. B16F10 cells. Control melanoma cells incubated in the dark remained viable. The growth of the blue light irradiated cells rapidly slowed after illumination as monitored by MTT colorimetric assay. The number of dead cells in the blue light irradiated group from D0 to D4 was also significantly greater than in the control group incubated in the dark (p<0.0001) (Figure 1A). No marked effects were noticed with the B16F10 cells after red light illumination in comparison with the control cells (p=0.29). The rate of mortality of the cells exposed to blue light and the cells exposed to red light was significantly different from D0 to D4 (p<0.0002) (Figure 1B). No regrowth was observed during the following days.

EJG cells. Blue light significantly increased endothelial cell death 24 h after irradiation in comparison to control cells (p<0.0001). Lesser red light phototoxicity was observed for the capillary endothelial cells compared to blue light toxicity (Figure 2A). No dead cells were noted after red light exposition but a significant difference in growth rate between exposed and unexposed EJG cells was noted (p=0.05).

Lipid peroxidation in B16F10 cells illuminated by blue light with two fluences 10J/cm^2 or 20J/cm^2. The LPO in the B16F10 cells illuminated at 10J/cm^2 or 20J/cm^2, medium alone and B16F10 without illumination was analyzed. The blue light illumination, at 10J or 20J/cm^2 did not affect the LPO values. No oxidative damage was found after blue light illumination, (Figure 2B).

Temperature. The temperature before and at the end of blue light illumination was recorded. A negligible increase from 26˚C±1.5˚C to 28˚C±1.3˚C, was recorded.

Clinical presentation. An 81-year-old man without any past medical history was referred to the Department of Dermatology in October 1999 for a malignant melanoma on his right thumb. His thumb was amputated in order to ensure

adequate margins for surgical removal of the melanoma. Histological analysis confirmed the diagnosis of malignant melanoma, (Breslow index: 3.8mm, Clark IV staging). Chest X-ray and abdominal ultrasound examination were normal at initial staging. Two years later, skin metastasis appeared on his right forearm and chest computed tomography demonstrated pulmonary metastasis. Chemotherapy with dacarbazine was started for a total of four courses. Unfortunately, he developed other pulmonary metastases. Seven courses of chemotherapy with cisplatin and vindesin were administered and the patient obtained tumour reduction between 20% and 50% of the pulmonary localizations, and he was followed up every 3 months. One year later, he was readmitted to our department for recurring widespread subcutaneous metastases on his right forearm with right axillary node involvement. After therapeutic lymph node dissection, treatment was completed by local nodal radiotherapy and interferon alfa 2b at a dose of three million IU per week for three months. However, on physical examination, several subcutaneous melanoma tumours were noted on his arm and forearm. Chest X-ray and abdominal-pelvic ultra-sound scan revealed no metastases except for the known pulmonary metastases. He was then treated with chemotherapy consisting of four cycles with cisplatin and vindesin. Clinical examination and chest X-ray showed progression with lesions slowly increasing in size. New chemotherapy with belustin for seven courses was started. All the subcutaneous metastases were haemorrhagic and thick. Simultaneously, blue light phototherapy was started on the anterior region of the right forearm. The 450 nm therapeutic light was generated by a Waldman lamp. The patient underwent one daily irradiation of 20 J/cm^2 5 days a week for 2 weeks. The diameter of metastasis was 2.8×3 cm before illumination and 2×2.4 cm after.

The adjacent skin was evaluated for erythema, oedema, blisters, necrosis and pain. The time and location of residual tumours and the development of new tumours were recorded. Photographs were taken routinely for more accurate comparison.

During the course of irradiation, the bleeding stopped rapidly and tumour growth was also interrupted in comparison to an unirradiated tumour on his right forearm (Figures 3A-3B). Necrosis tumour was observed 24 h after illumination. The size decreased to only 2 mm after 5 day and 7 mm after 2 weeks. On clinical examination, necrosis was observed. After two weeks, blue light phototherapy was stopped. One month later, recurrent bleeding and widespread tumour development were observed.

Discussion

This study confirmed that blue light therapy was effective in the destruction of melanoma B16F10 cells *in vitro*. We postulated that cell growth was inhibited because surviving cells did not continue to divide during the four days following illumination.

However, red light illumination was not effective for the melanoma cells. On the other hand, red light therapy was phototoxic on the endothelial cells. The difference between exposed endothelial cells and unexposed endothelial cells was significant during red light therapy. These findings suggested that the association of red light and photosensitizer might be a good combination for the destruction of vessels or tumoural vascularization.

Dramatic phototoxicity of blue light on the endothelial cells was noted. For this reason, blue light therapy was carried out on a patient and promising results were obtained. Clinical superficial necrosis of subcutaneous metastasis was observed 24 h after illumination. Also the bleeding stopped immediately after exposure to blue light.

Ohara *et al*. cells using a light-emitting diode (LED), reported that blue light, one of the primary colours composing the visible spectrum inhibited the growth of B16 melanoma (4). Their results indicated that blue light therapy exerted cytostatic effects but did not report any cytocidal action on B16F10 melanoma cells. These authors suggested that the inhibitory effect of blue light on the growth of B16 melanoma cells may result from an effect on the cell cycle, specifically, an inhibition of progression from G1 to S-phase and a prolongation of the M-phase (4'. In their study, no phototoxicity of red light was noted. Similar findings were obtained with the human leukaemic cell line HL60, where cell growth was found to be significantly suppressed following exposure to blue light, whereas the growth of normal lymphocytes was unaffected (8). The same team showed that exposure to blue light affected the potential of tumour cells to metastasize to the lung (9). These interesting results suggested that blue light therapy could be a good approach to the treatment of skin tumours. In the present study, blue light showed greater and more rapid cytotoxic effects than the blockage of the cell cycle demonstrated by Ohara *et al*. Similary to Godley *et al*., we suggest that in non-pigmented epithelial cells, the cytotoxic effect of blue light is caused by different types of damage (9).

Blue light exposure also significantly reduced the incidence and number of papillomas induced by TPA (12-*O*-tetradecanoylphorbol-13-acetate) application in v-Ha-ras transgenic mice (11). Therefore, skin tumours are logically one of the targets for treatment by blue light exposure, especially when tumours are numerous, and at the beginning of their evolution, when difficult to excise surgically, but readily accessible to external light exposure.

The blue light used for phototherapy in the present study was probably more effective than day light because a strong intensity narrow band of blue light was used which might correspond to the peak absorption wavelength at which an unidentified substance is modified (12). We suggest that a so far, undetermined molecular mechanism provokes a failure or a break in the cell cycle, depending on the intensity of

light, thus inducing cellular death. Data from Ohara *et al*. suggested that blue light suppressed melanin formation following repeated UVB exposure (13).

Moreover, riboflavin, contained in the DMEM culture medium, increased the effect of blue light inhibition of B16 melanoma growth by producing free radicals (14). Lockwood *et al*. noted that blue light generated ROS differently in tumour and normal epithelial cells (5). These data are interesting and suggest that a combination of blue light with photosensitizer might increase the efficacy of PDT even more than red light. Blue light is better tolerated and is strongly absorbed by porphyrins. Blue light could have a synergic action with porphyrins during PDT despite its low skin penetration.

Our results contradict further papers in which the authors report that the exposure of cells to blue light led to an increase in proliferation in human uveal melanoma cells compared to the control (15). There is confusion between visible blue light (450 nm to 490 nm) and ultraviolet (UV) (100 nm to 400 nm), wavelength is near and irradiation spectrum was not described in few articles. It is probably because there is a little UV in blue light. This confusion leads discordance in furthers articles. Di Cesare *et al*. reported that blue light exposure might influence the progression of uveal melanoma but no spectrum of blue light have noted (16).

The molecular mechanism of blue light action, including photosensitization, perturbation of the cell cycle and cell death by apoptosis or necrosis, remains to be elucidated. The role of blue light illumination in human melanoma deserves additional studies. The extent and suddenness of blue light induced cell death most likely suggested that death resulted from necrosis provoked by massive ROS production rather than by apoptosis. The aim of our future work is to investigate this further. In the present study, lipid peroxides were evaluated in cellular extracts of illuminated B16F10 cells as parameters of oxidative stress but no lipid peroxidation stress was observed with a $10J/cm^2$ or $20J/cm^2$. Thus it is suggested that another mechanism might explain the cell death.

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