Morphological Study in B16F10 Murine Melanoma Cells after Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG)

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ABSTRACT. Photodynamic hyperthermal therapy (PHT) with indocyanine green (ICG) is a combination of photodynamic therapy (PDT) and hyperthermia (HT). The low toxicity of ICG with an absorption wavelength of 700–800 nm is thought to make it a good candidate as a photosensitizer for PHT. Upon irradiation, ICG produces oxygen radicals and generates heat. The optimal concentration of ICG and the PHT post-irradiation time effects were evaluated by the cytotoxicity of the treatment on B16F10 murine melanoma. The cytotoxicity of PHT was determined based on the morphology of apoptotic and necrotic cells under phase-contrast microscope, confocal laser scanning microscope (CLSM) with DAPI and Annexin V-FITC/PI double staining, and cell surface structure evaluation by scanning electron microscopy (SEM). The use of ICG at a concentration of 150 μ M was selected, as cell proliferation was inhibited from 0 to 24 hr post-PHT with a 3-fold decrease in cell viability ($P < 0.001$) compared to the control group. A morphological observation revealed apoptotic and some degree of necrotic features in the PHT-treated cells.

KEY WORDS: apoptosis, indocyanine green, morphology, photodynamic hyperthermal therapy.

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Indocyanine green (ICG) is a water soluble anionic tricarbocyanine dye with an absorption wavelength of 700–800 nm [11]. The United States Food and Drug Administration (FDA) has approved ICG for the assessment of cardiac output, hepatic function and blood flow, and for ophthalmic angiography. The principle advantage of ICG is its low toxicity [18]. It is known that ICG induces heat in response to light with a wavelength of 800 nm [35]. Based on this property, many *in vivo* and *in vitro* experiments have been performed [8, 34]. Hirano *et al.* [17] first demonstrated that ICG could induce singlet oxygen formation in response to light with a wavelength between 600–800 nm, which means that ICG has properties that could make it useful as a photosensitizer during photodynamic therapy (PDT) [2]. We noticed this property of ICG and developed a new cancer therapy based on the properties of ICG and a broad band light source that emits light of 600–800 nm to create a combination modality utilizing both photodynamic therapy (PDT) and hyperthermia. We called this therapy photodynamic hyperthermal therapy (PHT).

We herein investigated the mechanism(s) underlying the anti-tumor effects of PHT. The objective of this study was to investigate the ability of PHT to induce cell death and inhibit the proliferation of the murine melanoma cell line, B16F10 by morphological techniques.

MATERIALS AND METHODS

Cell culture and ICG preparation: The murine melanoma cell line, B16F10 (Riken Cell Bank, Tsukuba, Japan), was maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 100 units/ml of penicillin-streptomycin-neomycin solution at 37°C in a humidified incubator with 5% CO₂. Indocyanine green (Diagnogreen, Daiichi Sankyo, Tokyo, Japan) was dissolved in DMEM at concentrations of 50, 150, 250, 350, and 500 μ M.

Light source: In this study, the linear polarized infrared device (wavelength: 600–1,600 nm) with energy output of 5 W, Super Lizer™ model PX (SL, IKEN, Tokyo, Japan) was used as a light source.

In vitro treatment and cell viability assay: A cell counting kit-8 (CCK-8) (Dojindo Laboratories, Kumamoto, Japan) was used to evaluate the cell viability after PHT. To determine the optimal concentration of ICG, cells (1×10^4 cells) were seeded in 96-well cell culture plates and kept in the incubator at 37°C overnight. The cells were then treated with a range of ICG concentrations (0, 50, 150, 250, 350,

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and 500 μM) in the culture media. After 24 hr, the media were discarded and cell cultures were washed twice with PBS. Fresh media (200 μl) were added, and the cell cultures were subjected to SL irradiation for 3 min, stationed at a 3 cm distance from the targeted surface, with power density at 0.33 W/m^2 and energy dose at 59.4 J/m^2 . The irradiated cells were cultured for 3, 6, 12, or 24 hr with avoidance of light, while the test was performed immediately for the 0 hr group. Ten microliters of CCK-8 was added to each well post-PHT and incubated for 4 hr in an incubator. The absorbance was measured with a microplate reader (ImmunoMini NJ-2300, System Instruments, Tokyo, Japan) at 405 nm. To compare the cell viability between treatments, the cells were divided into 4 groups; control (no treatment), ICG only, light only and PHT. The cells in the ICG only and PHT groups were incubated with 150 μM of ICG for 24 hr. At the same time, the media of control and light groups were replaced with fresh media. The next day, the media in the light and PHT groups were changed to new media, and the cells were irradiated at 59.4 J/cm^2 for 3 min, stationed at a 3 cm distance from the targeted surface. A cell viability assay was performed as described above. The temperature of the media in the light and PHT groups was controlled at 43°C. The cell culture dishes for these 2 groups were positioned on the surface of crushed ice, and thermocouple probes (Anritsu Meter Co., Ltd., Tokyo, Japan) were placed in the media to monitor the temperature. The temperature of the control and ICG only groups were maintained at the incubator temperature (37°C).

Morphologic observation after PHT: After PHT with 150 μM of ICG, the cell morphology was assessed by a phase contrast microscope (Nikon Eclipse TS100, Nikon, Tokyo, Japan) and evaluation by a confocal laser scanning microscope (CLSM) (Olympus FluoView® FV10i, Olympus, Tokyo, Japan.) at 0, 3, 6, 12, and 24 hr post-PHT. For 4',6-diamidino-2-phenylindole dihydrochloride (DAPI) staining, cells (1×10^5) were grown on glass bottom culture dishes and were fixed with 3.7% formaldehyde for 10 min, then permeabilized with 0.2% Triton X-100 and stained with 10 $\mu\text{g}/\text{ml}$ DAPI for 5 min at 37°C. The cells were washed with phosphate-buffered saline (PBS) and examined. For Annexin V and Propidium Iodide (PI) staining, the cells were grown on glass bottom culture dishes, and after treatment, 100 μl of Annexin V incubation reagent (TACS® Annexin V-FITC Kit, Trevigen, Gaithersburg, MD, U.S.A.) which consisted of 10 μl 10 $\times$ Binding buffer, 10 μl PI, 1 μl fluorescein isothiocyanate (FITC)-conjugated Annexin V and 79 μl distilled water, were spread to cover the cells, which were incubated in the dark for 15 min. Samples were then washed twice and observed immediately.

The treated cells were also examined by scanning electron microscopy (SEM) as follows: The monolayer cells which were grown on 13 mm round cover slips were washed twice with PBS. The cells were fixed with 2.5% glutaraldehyde in 0.1M PBS (pH 7.2) at 4°C for 1 hr. Then the cells were rinsed with cold PBS and fixed with 1% osmium tetroxide at room temperature for 20 min. After dehydrating them with gradient ethanol, the specimens were immersed

in tertiary-butyl alcohol and subjected to critical point drying. The specimens are mounted on aluminum stubs with adhesive tabs and sputter coated with gold and palladium alloy (E101 Ion Sputter, Hitachi, Tokyo, Japan). The specimen surface structures were observed by SEM (S-2380N, Hitachi, Tokyo, Japan).

Statistical analyses: The data were presented as the means from at least 3 independent experiments, and the error bars represent the standard error of the mean (SEM). The statistical significance was evaluated using an ANOVA, and a P -value <0.05 was considered to be statistically significant. All statistical tests were performed using the SPSS for Windows software program (SPSS version 16.0, Chicago, IL, U.S.A.).

RESULTS

The effects of PHT on cell proliferation: To evaluate the effects of PHT on the B16F10 cells, different concentrations (50, 150, 250, 350, and 500 μM) and durations of exposure (0, 3, 6, 12 and 24 hr post-treatment) were used. As shown in Fig. 1I, the cell proliferation was inhibited from 0 to 24 hr post-PHT at the 150 μM concentration of ICG. The concentrations of 250, 350 and 500 μM showed the same pattern of effects on the cell proliferation as 150 μM . At 50 μM , PHT had no inhibitory effect on the cell proliferation at the designated time points. The 150 μM concentration of ICG was therefore selected for further experiments. Figure 1J shows the immediate responses of different treatment groups at 0 hr. PHT led to a 3-fold inhibition of cell viability ($P < 0.001$) compared to the control group.

Morphological observation by phase contrast microscope: Under phase contrast microscope, the cells in the control group were spindle-shape, in close contact with neighboring cells, and adhered to the surface of the culture flask (Fig. 1A). The phenotypic characteristics of post-PHT cells (Fig. 1D–G) showed cells shrinkage, with rounded or irregular cell outlines and many vacuole-like structures in the cytoplasm. The morphological changes were detected immediately after PHT. Twenty-four hours post-PHT, there was evidence that cells had disappeared, mainly due to detachment from the cell culture dish or disintegration into cellular fragments (Fig. 1H). Cells in the ICG and light only groups showed a loss of contact between cells, but no vacuole formation in the cytoplasm (Fig. 1B and 1C).

Morphological observation by confocal laser scanning microscope: In order to determine whether the anti-proliferative effect of PHT was related to the induction of apoptosis, the morphological features of cell apoptosis were investigated with DAPI and Annexin V and PI double staining. Figure 2B shows the presence of condensation at the periphery of the nucleus as early as 0 hr post-PHT, as well as evidence of cell shrinkage. The nuclear area stained by DAPI also decreased, as indicated by the pinpoint appearance over time. This is a sign of nuclear fragmentation, which is in contrast to control cells, which showed full nuclear staining and a smooth nuclear perimeter (Fig. 2A). The cell outline became convoluted and formed ex-

Fig. 1. PHT inhibits cell proliferation in B16F10. (A) control: without treatment. (B) ICG. (C) Light. (D) 0 hr post-PHT. (E) 3 hr post-PHT. (F) 6 hr post-PHT. (G) 12 hr post-PHT. (H) 24 hr post-PHT. The effect of PHT on cell growth, phase contrast image (200 $\times$). (I) The effects of PHT with different concentration of ICG at 50, 150, 250, 350, and 500 μ M on cell viability between 0–24 hr. (J) Immediate effect of PHT on cell viability at 0 hr post- PHT in comparison to control, ICG only and SL only groups. Results represent the mean $\pm$ SE (n=3). * indicates a significance difference from control group, $P < 0.001$.

tensions with generalized vacuolization in the cytosol and a decrease in the cytoplasm:nucleus ratio in the post-PHT group (Fig. 2C and 2D). Figure 2E and 2F show evidence of black pigment release, which is an indication of cell death.

Figure 3 shows the results of the post-PHT time-dependent study on the apoptosis rate with Annexin V and PI double staining for *in situ* detection. The cytoplasm was stained green with Annexin V and the nucleus appeared red due to PI staining. Figure 3A shows that there was minimal greenish staining of live cells. From 0 to 24 hr post-PHT, the majority of cells gradually changed from early apoptosis to late apoptosis and necrosis. Figure 3B and 3C showing early apoptosis, had cells that were only stained green, while Fig. 3D and 3E showing late apoptosis cells were stained with both Annexin V in the cytoplasm and PI in the nucleus. In Fig. 3F, a mixture of late apoptosis and necrosis was present, which was indicated by the red staining in the nucleus.

Morphological observation by scanning electron microscope: The SEM examination revealed that the untreated cells were spindle-shaped, with a smooth and intact plasma

membrane (Fig. 4A). Figure 4B and 4C show no evidence of cell shrinkage, rounding or bleb formation after ICG and light only treatments, respectively. However in the ICG group, the cell surface was wrinkled, but with an intact cell surface. At 0 hr post-PHT, the cells exhibited shrinkage and blebbing of the plasma membrane (Fig. 4D). In addition, they lost the plasma membrane connections with neighboring cells. At 3 hr post-PHT, the cells showed severe surface alterations (Fig. 4E). The cells became rounded and more blebs and vesicles were formed on the surface. Figure 4F, 4G and 4H showing the results at 6, 12 and 24 hr post-PHT, respectively, showed severe changes in the cell shape and surface structure. The rupture of plasma membranes and cytoplasmic damage were visualized, and there was flattening with attachment of cell remnants, indicating necrotic succession.

DISCUSSION

Melanoma is the most aggressive form of skin cancer, and is resistant to the currently-used cancer therapeutic

Fig. 2. PHT induced changes in nucleus morphology, stained with DAPI. Representative images of (A) control. (B–F) Post PHT with time indicated. (B) $\Rightarrow$: blebbing, $\Rightarrow$: perinuclear condensation, $\Rightarrow$: cytosol vacuolization. Confocal laser scanning microscopy images.

Fig. 3. PHT induced phosphatidylserine externalization which an indicator of early apoptosis in B16F10. Cytoplasm was stained green with Annexin V-FITC and nucleus appeared red with PI. Representative images of (A) control. (B–D) Annexin V-FITC positive cells indicates early apoptosis. (E) Combination of Annexin V-FITC apoptotic cells and PI implies late apoptosis. (F) Mostly PI stained cells denotes necrotic cells. Confocal laser scanning microscopy images.

Fig. 4. PHT induced cellular surface alterations in B16F10 cells. Representative images of (A) control: without treatment. (B) ICG. (C) Light. (D) 0 hr post-PHT, blebs indicated with 2 white arrows. (E) 3 hr post-PHT. (F) 6 hr post-PHT. (G) 12 hr post-PHT. (H) 24 hr post-PHT. Scanning electron microscopy images.

modalities [33]. We chose to evaluate B16F10 cells, because this cell line is used as a mouse model for human melanoma studies [28], and we hope that this PHT method can be applied in both the human and veterinary fields. Melanotic melanomas have a poor response to conventional PDT. The reason for this is that melanin absorbs light over the entire wavelength region used for PDT (400–750 nm) [25]. This creates competition between the melanin and the photosensitizers commonly used in PDT. Therefore, the efficacy of the photosensitizing process is minimized. In our study, we used PHT with a near infra red (NIR) light source and ICG to produce heat and oxygen radicals when the compound was irradiated at 600–800 nm. This was hypothesized to be more effective than conventional PDT, because 800 nm is not in the absorption range of melanin.

ICG and other photosensitizers (PSs) can be uptaken by normal and cancer cells *in vitro*, followed by photooxidation if irradiated [2, 32]. However, *in vivo* PSs selectively accumulate in the tumor due to the absence of lymphatic drainage and leaky blood vessels from bizarre neovascularization [7]. PSs are attached to molecules that have affinity

to receptors expressed on tumors [19]. Elevation of low density lipoprotein (LDL) receptors on cancer cells to mediate the endocytosis of cholesterol-rich LDL into actively dividing cancer cells for synthesizing new membranes enhanced the PSs accumulation [21]. ICG was found to accumulate in solid tumor after systemic and intratumoral application [1]. When reactive oxygen species is produced therapeutically from PSs irradiation which accumulated in tumor cells, tumor cells reach the death threshold earlier and thus kill the tumor cells [37]. Making this treatment tumor specific.

In the present study, we found that PHT can suppress the proliferation of melanoma cells in a dose-dependent manner *in vitro*. A morphological observation was performed to explore whether the cytotoxic effect was related to the induction of apoptosis. The morphological changes that characterize apoptosis are mainly cell shrinkage and rounding [29]. At 0 to 24 hr post-PHT, a large number of rounded cells were observed by a phase contrast microscopy. Apoptotic morphological changes, including cell shrinkage, increased cytoplasm granularity and cell fragmentation were observed as reported previously [20, 42]. In the morpho-

logical study, it was suggested that PHT induced acute and persistent apoptosis in the B16F10 cells.

Furthermore, alterations of the nuclear morphology were observed in a time-dependent manner by DAPI staining with CLSM. The early changes observed in this study were the condensation of nuclear chromatin along the perimeter of the nucleus [13], cytoplasmic shrinkage, vacuoles formation in the cytosol and separation from surrounding cells. This was followed by nuclear fragmentation, but no apoptotic bodies were observed due to limited length of the time course designed for this study. According to Aoyama *et al.* [3], condensation of the nucleus was observed at 6 hr, fragmentation at 24 hr and apoptotic body formation at 48 hr in astrocyte acidosis. Based on the presented morphological evidence, Annexin V and PI double staining by CLSM showed that PHT could induce apoptosis. Positive uptake of Annexin V indicated the presence of phosphatidylserine (PS) externalization [38]. PS is primarily observed on the inner surface of the lipid bilayer of the plasma membrane facing the cytosol. The plasma membrane of cells that are undergoing apoptosis will have a break up of the phospholipid asymmetry and expose PS on the surface, which an indicator of early apoptosis [14]. The cells evaluated after longer post-PHT times were positive for PI, indicating the presence of a loss of membrane integrity, which provided greater access to the inside of the cells [36]. Thus, double labeling was used to help differentiate between early and late apoptotic or necrotic events.

The apoptotic morphological changes observed by SEM were mainly cell shrinkage and a loss of cell membrane integrity. Our results indicate that the apoptotic cells presented membrane blebbing or zeiosis, which has been described in many other cells types [6, 39, 40] following PDT. Most of the apoptotic cells following PHT exhibited a loss of cellular adhesion and were detached from the cover slip. Hence, the number of cells observed after treatment was lower in the treated cells compared to the untreated group even though the initial cell inoculation number was the same (1×10^6 cells).

In this study, the SEM analysis allowed for a detailed examination of cell surface changes and to distinguish the different types of cell death. From the SEM, we found that apoptosis can be induced by PHT. However, some cells might undergo necrosis due to the morphological surface rupture observation. The possible explanation for the presence of both apoptotic and necrotic cell death is the different uptake rates of ICG and the individual cell cycle stages when treatment was initiated. The occurrence of necrosis events during PHT may also be related to the melanin production in the melanoma cells [22]. Pigmented cells have been reported to localize the photosensitizer in membrane-bound organelles known as melanosomes [15]. Therefore, the ICG which accumulates in the membranes, and upon activation, causes the leakage of melanin derivatives, thereby inducing necrosis. Our study showed that bleb or vesicle formation is an immediate feature of PHT, and loss of membrane integrity in some cells later.

In vitro studies with various cell lines have reported the

induction of apoptosis by hyperthermia, although the temperatures used have varied, with a longer duration of treatment, and much later detection of apoptotic changes being reported. Delpino *et al.* [10] exposed a human melanoma cell line (M-14) to 42°C for 4 hr with no obvious morphological changes detected, but a computerized image analysis demonstrated a significant enlargement of both nuclei and nucleoli. Rong and Mack [30] heated the Dunn osteosarcoma cell line at 43.5°C for 1 hr, and noted that the cells demonstrated apoptotic morphology, such as condensed chromatin and fragmented nuclei, at 6 hr post-treatment. Other studies induced hyperthermia at temperatures ranging from 42 to 45°C with a duration of treatment between 30 and 90 min. Various morphological changes were observed, including plasma membrane changes, vacuolated cytoplasm and condensed nuclei [4, 23, 31, 41]. Thus, cancer cell lines appear to be sensitive to hyperthermia, although the optimal treatment varied among the different cell lines [16].

ICG-PDT with a continuous wave laser (e.g., diode laser) was documented in various *in vitro* studies. MCF-7 human breast cancer cells were treated with a combination of ICG-PDT with cisplatin, and the temperature was fixed at 35°C with a light dose at 25 J/cm² and 20 µM of ICG. Their 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide method results after 5 hr of ICG-PDT showed that the reduction in cell viability was greater for cisplatin plus PDT than when the two treatments administered separately [9]. A cell viability analysis was performed after 5 hr of ICG-PDT in comparison to the PHT in our study with the presence of hyperthermia at 43°C, and the cell viability assessment showed immediate significant reduction in comparison to the control group even without combination with a chemotherapy drug. In another study, Sk-Mel-28 human skin melanoma cells were irradiated at 788 nm with 150 µM ICG, with the length of irradiation ranging from 15 to 60 min [26]. In this study, the cell viability was reduced and apoptosis was induced as the application time increased. The cell viability was analyzed 48 hr post-treatment, and the surviving fraction of the irradiated cells was found to have decreased. In studies of HT-29 human colon cancer cells, irradiation was performed at 805 nm with a light dose of 30 J/cm², along with 500 µM ICG, and the temperature was measured to ensure that no hyperthermic conditions were imposed on the cells. The cell viability was significantly decreased after 24 hr post ICG-PDT in that study [5]. In our current study with PHT, a lower molar concentration was used, and a significant decrease in the cell viability was rapidly achieved with the imposition of hyperthermia at 43°C.

Gratz *et al.* [12] reported that the singlet oxygen yielded after excitation of ICG was low, and that a higher concentration of ICG or light fluorescence rate was needed compared to when porphyrin was used as the photosensitizer. The apoptotic morphology of PDT and hyperthermia described previously [24, 27] share common features with the cells in our study treated using PHT. The synergistic effect of PHT in this study gives advantages over PDT and hyperthermia.

For example, the short irradiation duration and immediate effect on cell proliferation in the B16F10 cells proceeded to the detection of apoptotic and necrotic morphology within 24 hr. In addition, PHT required a lower molar concentration of ICG and a lower light dose to achieve a significant reduction in cell viability. Furthermore, PHT enhanced the susceptibility of B16F10 cells to death.

In summary, PHT has a cytotoxic effect on B16F10 cells. Apoptotic and necrotic morphological changes were observed. The current study demonstrated that PHT inhibited the proliferation of B16F10 cells by inducing apoptosis, and later some degree of necrosis.

REFERENCES

- Abels, C. 2004. Targeting of the vascular system of solid tumours by photodynamic therapy (PDT). *Photochem. Photobiol. Sci.* **3**: 765–771. [Medline] [CrossRef]
- Abels, C., Fickweiler, S., Weiderer, P., Bäuml, W., Hofstädter, F., Landthaler, M. and Szeimies, R. M. 2000. Indocyanine green (ICG) and laser irradiation induce photooxidation. *Arch. Dermatol. Res.* **292**: 404–411. [Medline] [CrossRef]
- Aoyama, K., Burns, D. M., Suh, S. W., Garnier, P., Matsumori, Y., Shiina, H. and Swanson, R. A. 2005. Acidosis causes endoplasmic reticulum stress and caspase 12-mediated astrocyte death. *J. Cereb. Blood Flow Metab.* **25**: 358–370. [Medline] [CrossRef]
- Arancia, G., Crateri Trovalusci, P., Mariutti, G. and Mondovì, B. 1989. Ultrastructural changes induced by hyperthermia in Chinese hamster V79 fibroblasts. *Int. J. Hyperthermia* **5**: 341–350. [Medline] [CrossRef]
- Bäuml, W., Abels, C., Karrer, S., Weiss, T., Messmann, H., Landthaler, M. and Szeimies, R. M. 1999. Photo-oxidative killing of human colonic cancer cells using indocyanine green and infrared light. *Br. J. Cancer* **80**: 360–363. [Medline] [CrossRef]
- Bednars, N., Zawacka-Pankau, J. and Kowalska, A. 2007. Protoporphyrin IX induces apoptosis in HeLa cells prior to photodynamic treatment. *Pharmacol. Rep.* **59**: 474–479. [Medline]
- Castano, A. P., Demidova, T. N. and Hamblin, M. R. 2005. Mechanisms in photodynamic therapy: part three—photosensitizer pharmacokinetics, biodistribution, tumor localization and modes of tumor destruction. *Photodiagn. Photodyn. Ther.* **2**: 91–106. [CrossRef]
- Chen, W. R., Adams, R., Higgins, A., Bartels, K. and Nordquist, R. 1996. Photothermal effects on murine mammary tumors using indocyanine green and an 808-nm diode laser: an in vivo efficacy study. *Cancer Lett.* **98**: 169–173. [Medline]
- Crescenzi, E., Varriale, L., Iovino, M., Chiaviello, A., Veneziani, B. M. and Palumbo, G. 2004. Photodynamic therapy with indocyanine green complements and enhances low-dose cisplatin cytotoxicity in MCF-7 breast cancer cells. *Mol. Cancer Ther.* **3**: 537–544. [Medline]
- Delpino, A., De Santis, M., Sudano, L., Perrone Donnorso, R. and Ferrini, U. 1989. Morphometric analysis of hyperthermia-induced nuclear and nucleolar size modifications in a human melanoma cell line. *Diagn. Cytopathol.* **5**: 427–431. [Medline] [CrossRef]
- Fickweiler, S., Szeimies, R., Bäuml, W., Steinbach, P., Karrer, S., Goetz, A. E., Abels, C., Hofstädter, F. and Landthaler, M. 1997. Indocyanine green: intracellular uptake and phototherapeutic effects in vitro. *J. Photochem. Photobiol. B* **38**: 178–183. [Medline] [CrossRef]
- Gratz, H., Penzkofer, A., Abels, C., Szeimies, R. M., Landthaler, M. and Bäuml, W. 1999. Photo-isomerisation, triplet formation, and photodegradation dynamics of Indocyanine green solutions. *J. Photochem. Photobiol. A* **128**: 101–109. [CrossRef]
- Häcker, G. 2000. The morphology of apoptosis. *Cell Tissue Res.* **301**: 5–17. [Medline] [CrossRef]
- Hanshaw, R. G., Lakshmi, C., Lambert, T. N., Johnson, J. R. and Smith, B. D. 2005. Fluorescent detection of apoptotic cells by using zinc coordination complexes with a selective affinity for membrane surfaces enriched with phosphatidylserine. *Chem. Biol. Chem.* **6**: 2214–2220. [Medline] [CrossRef]
- Hearing, V. J. 2005. Biogenesis of pigment granules: a sensitive way to regulate melanocyte function. *J. Dermatol. Sci.* **37**: 3–14. [Medline] [CrossRef]
- Hildebrandt, B., Wust, P., Ahlers, O., Dieing, A., Sreenivasa, G., Kerner, T., Felix, R. and Riess, H. 2002. The cellular and molecular basis of hyperthermia. *Crit. Rev. Oncol. Hematol.* **43**: 33–56. [Medline] [CrossRef]
- Hirano, T., Kohno, E., Gohto, Y. and Obana, A. 2007. Singlet oxygen generation by irradiation of Indocyanine green (ICG) and its effect to tissues. *JSLSM* **28**: 122–128. [CrossRef]
- Hope-Ross, M., Yannuzzi, L. A., Gragoudas, E. S., Guyer, D. R., Slakter, J. S., Sorenson, J. A., Krupsky, S., Orlock, D. A. and Puliafito, C. A. 1994. Adverse reactions due to indocyanine green. *Ophthalmology* **101**: 529–533. [Medline]
- Iyer, A. K., Greish, K., Seki, T., Okazaki, S., Fang, J., Takeshita, K. and Maeda, H. 2007. Polymeric micelles of zinc protoporphyrin for tumor targeted delivery based on EPR effect and singlet oxygen generation. *J. Drug Target.* **15**: 496–506. [Medline] [CrossRef]
- Janson, V., Behnam-Motlagh, P., Henriksson, R., Hörstedt, P., Engström, K. G. and Grankvist, K. 2008. Phase-contrast microscopy studies of early cisplatin-induced morphological changes of malignant mesothelioma cells and the correspondence to induced apoptosis. *Exp. Lung Res.* **34**: 49–67. [Medline] [CrossRef]
- Kessel, D. 1992. The role of low-density lipoprotein in the bio-distribution of photosensitizing agents. *J. Photochem. Photobiol. B* **14**: 261–262. [Medline] [CrossRef]
- Koo, H. M., VanBrocklin, M., McWilliams, M. J., Leppla, S. H., Duesbery, N. S. and Woude, G. F. 2002. Apoptosis and melanogenesis in human melanoma cells induced by anthrax lethal factor inactivation of mitogen-activated protein kinase. *Proc. Natl. Acad. Sci. U.S.A.* **99**: 3052–3057. [Medline] [CrossRef]
- Liu, H. Y., Zhao, K., Zhou, M. M., Wang, C., Yu, J. E. and Liu, J. X. 2010. Cytoprotection of vitamin E on hyperthermia-induced damage in bovine mammary epithelial cells. *J. Therm. Biol.* **35**: 250–253. [CrossRef]
- Luchetti, F., Canonico, B., Della Felice, M., Burattini, S., Battistelli, M., Papa, S. and Falcieri, E. 2003. Hyperthermia triggers apoptosis and affects cell adhesiveness in human neuroblastoma cells. *Histol. Histopathol.* **18**: 1041–1052. [Medline]
- Ma, L. W., Nielsen, K. P., Iani, V. and Moan, J. 2007. A new method for photodynamic therapy of melanotic melanoma effects of depigmentation with violet light photodynamic therapy. *J. Environ. Pathol. Toxicol. Oncol.* **26**: 165–172. [Medline]
- Mamoon, A. M., Gamal-Eldeen, A. M., Ruppel, M. E., Smith, R. J., Tsang, T. and Miller, L. M. 2009. In vitro efficiency and mechanistic role of indocyanine green as photodynamic therapy agent for human melanoma. *Photodiagnosis Photodyn.*

- Ther.* **6**: 105–116. [Medline] [CrossRef]
27. Noodt, B. B., Berg, K., Stokke, T., Peng, Q. and Nesland, J. M. 1996. Apoptosis and necrosis induced with light and 5-aminolaevulinic acid-derived protoporphyrin IX. *Br. J. Cancer* **74**: 22–29. [Medline] [CrossRef]
 28. Overwijk, W. W. and Restifo, N. P. 2001. B16 as a mouse model for human melanoma. pp. 20.1.1–20.1.29. In: *Current Protocols in Immunology* (Coligan, J. E., Bierer, B. E. Margulies, D. H., Shevach, E. M. and Strober, W. eds.), John Wiley and Sons, New York.
 29. Rello, S., Stockert, J. C., Moreno, V., Gámez, A., Pacheco, M., Juarranz, A., Cañete, M. and Villanueva, A. 2005. Morphological criteria to distinguish cell death induced by apoptotic and necrotic treatments. *Apoptosis* **10**: 201–208. [Medline] [CrossRef]
 30. Rong, Y. and Mack, P. 2000. Apoptosis induced by hyperthermia in Dunn osteosarcoma cell line in vitro. *Int. J. Hyperthermia* **16**: 19–27. [Medline] [CrossRef]
 31. Shellman, Y. G., Howe, W. R., Miller, L. A., Goldstein, N. B., Mahanjan, R. L., LaRue, S. M. and Norris, D. A. 2008. Hyperthermia induces endoplasmic reticulum-mediated apoptosis in melanoma and non-melanoma skin cancer cells. *J. Invest. Dermatol.* **128**: 949–956. [Medline] [CrossRef]
 32. Skrivánová, K., Skorpíková, J., Svihálek, J., Mornstein, V. and Janisch, R. 2006. Photochemical properties of a potential photosensitizer indocyanine green in vitro. *J. Photochem. Photobiol. B* **85**: 150–154. [Medline]
 33. Soengas, M. S. and Lowe, S. W. 2003. Apoptosis and melanoma chemoresistance. *Oncogene* **22**: 3138–3151. [Medline] [CrossRef]
 34. Tseng, W. W., Saxton, R. E., Deganutti, A. and Liu, C. D. 2003. Infrared laser activation of indocyanine green inhibits growth in human pancreatic cancer. *Pancreas* **27**: e42–e45. [Medline] [CrossRef]
 35. Urbanska, K., Romanowska-Dixon, B., Matuszak, Z., Oszajca, J., Nowak-Sliwinski, P. and Stochel, G. 2002. Indocyanine green as a prospective sensitizer for photodynamic therapy of melanomas. *Acta Biochim. Pol.* **49**: 387–391. [Medline]
 36. Vermes, I., Haanen, C., Steffens-Nakken, H. and Reutelingsperger, C. 1995. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J. Immunol. Methods* **184**: 39–51. [Medline] [CrossRef]
 37. Wang, J. and Yi, J. 2008. Cancer cell killing via ROS: to increase or decrease, that is the question. *Cancer Biol. Ther.* **7**: 1875–1884. [Medline] [CrossRef]
 38. Yan, T., Seo, Y., Schupp, J. E., Zeng, X., Desai, A. B. and Kinsella, T. J. 2006. Methoxyamine potentiates iododeoxyuridine-induced radiosensitization by altering cell cycle kinetics and enhancing senescence. *Mol. Cancer Ther.* **5**: 893–902. [Medline] [CrossRef]
 39. Yow, C. M., Mak, N. K., Leung, A. W. and Huang, Z. 2009. Induction of early apoptosis in human nasopharyngeal carcinoma cells by mTHPC-mediated photocytotoxicity. *Photodiagnosis Photodyn. Ther.* **6**: 122–127. [Medline] [CrossRef]
 40. Yow, C. M., Wong, C. K., Huang, Z. and Ho, R. J. 2007. Study of the efficacy and mechanism of ALA-mediated photodynamic therapy on human hepatocellular carcinoma cell. *Liver Int.* **27**: 201–208. [Medline] [CrossRef]
 41. Zhou, J., Wang, X., Du, L., Zhao, L., Lei, F., Ouyang, W., Zhang, Y. and Liao, Y. 2011. Effect of hyperthermia on the apoptosis and proliferation of CaSki cells. *Mol. Med. Report* **4**: 187–191. [Medline]
 42. Ziegler, U. and Groscurth, P. 2004. Morphological features of cell death. *News Physiol. Sci.* **19**: 124–128. [Medline] [CrossRef]