Photodynamic Hyperthermal Therapy with Indocyanine Green (ICG) induces Apoptosis and Cell Cycle Arrest in B16F10 Murine Melanoma Cells

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ABSTRACT. We examined the effects of photodynamic hyperthermal therapy (PHT), which is a combination of photodynamic therapy (PDT) and hyperthermia (HT), on the apoptosis and cell cycle progression of murine melanoma B16F10 cells. The percentage of apoptotic cell was determined by flow cytometry using fluorescein isothiocyanate (FITC)-conjugated Annexin V and propidium iodide (PI) double staining. The cell cycle analysis was performed by PI staining with flow cytometry. The expression of cyclins and heat shock protein 70 (Hsp70) were examined by a Western blotting analysis. PHT induces death in b16F10 cells, and PHT-mediated apoptosis occurred acutely and persistently in vitro. Our study demonstrated that PHT using indocyanine green (ICG) and near infrared (NIR) light source induces apoptosis and G0/G1 cell cycle arrest in the B16F10 cells.

KEY WORDS: apoptosis, cell cycle arrest, indocyanine green, photodynamic hyperthermal therapy.


The aim of cancer therapy is to induce cell death without disturbing normal tissues. Cell death is divided into two main types, apoptosis and necrosis. We developed a new cancer therapy utilizing indocyanine green (ICG) and a broad band light source, to create a combination of photodynamic therapy (PDT) and hyperthermia (HT), called photodynamic hyperthermal therapy (PHT). ICG was used as the photosensitizer in this study, is a water soluble anionic tricarbocyanine dye with an absorption wavelength of 700–800 nm [5]. The principle advantage of ICG is its low toxicity [17] and the fact that it has already been approved by the United States Food and Drug Administration (FDA) for the assessment of cardiac output, hepatic function and blood flow, and for ophthalmic angiography.

PDT-mediated cell apoptosis has been reported in both in vivo and in vitro studies [12, 35, 38]. Other researchers have reported cell cycle arrest in different phases by PDT [20, 33, 42]. Furthermore PDT was able to initiate an immune response [9, 28, 29] and to generate cancer vaccine [21, 22]. Many studies of PDT using ICG as photosensitizer did not demonstrate cell cycle arrest. HT is a therapeutic procedure used to raise the temperature between 40 to 43°C to achieve a tumor killing effect [47]. Cancer cell growth inhibition with HT has been reported in numerous in vitro and in vivo studies [18, 31, 34]. However, researchers in PDT reported much later apoptosis detection and in HT required long duration of treatment. Hence, PHT may exert acute apoptosis with short duration of treatment, making PHT attractive treatment compared to PDT or HT.

ICG induces photo-oxidative cell killing mediated by singlet oxygen [1, 14] and heat generation [45]. However, there are several unclear points regarding how PHT can induce cell cycle arrest, and a quantitative evaluation of apoptosis and caspase family participation has not been performed.

The objective of this study was to investigate the effects of PHT on cell cycle arrest, apoptosis and heat shock protein 70 (Hsp70) expression in B16F10 melanoma cells.

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-cneomycin at 37°C in a humidified incubator with 5% CO₂. Indocyanine green (Diagnogreen, Daiichi Sankyo, Tokyo, Japan) was dissolved in DMEM at a concentration of 150
μM, which was determined by preliminary data.

Light source: In this study, a linear polarized near infrared device (wavelength: 600–1600 nm) average output 5 W, from Super Lizer™, model PX (SL, IKEN, Tokyo, Japan) was used as a light source. The energy dose delivered was 59.4 J/cm² with power density at 0.33 W/m², for 3 min irradiation and stationed at a 3 cm distance from the surface of the target. The temperature of the media in the light and PHT groups were controlled at 43°C. The cell culture dishes were positioned on the surface of crushed ice, and a thermocouple probe (Anritsu Meter Co., Ltd., Tokyo, Japan) was placed in the media to monitor the temperature.

Cell cycle analysis with propidium iodide (PI): The B16F10 cells were seeded in tissue culture dishes and incubated under standard conditions. The culture media was replaced with fresh media containing 150 μM ICG, and cells were cultured for 24 hr. After 24 hr, the media containing ICG were discarded. Cell cultures were washed twice with PBS and replaced with fresh media. The cells were then subjected to PHT at 59.4 J/cm² and collected at 0, 3, 6, 12 and 24 hr post-PHT. The cells were trypsinized and washed twice in ice cold PBS and stored at −20°C in 70% ethanol. Fixed cells were resuspended at a concentration of 1 × 10⁶ cells/ml with a staining solution consisting of 50 μg/ml PI (Sigma-Aldrich, St. Louis, MO, U.S.A.), 0.1% Triton X-100, 0.1 mM Ethylenediaminetetraacetic acid (EDTA) and 50 μg/ml RNase (Sigma-Aldrich) in PBS. The samples were then incubated for 30 min on ice. Flow cytometry was performed on a FACScal flow cytometer (BD Biosciences) and DNA histograms were analyzed with the FlowJo software program (Treestar Software, San Carlos, CA, U.S.A.).

Analysis of apoptosis by fluorescein isothiocyanate (FITC)-conjugated Annexin V and PI staining: The B16F10 cells were seeded in tissue culture dishes and incubated under standard conditions. The culture media was replaced with fresh media containing 150 μM ICG, and cells were cultured for another 24 hr. After 24 hr, the media containing ICG were discarded. Cell cultures were washed twice with PBS and replaced with fresh media. The cells were then subjected to PHT at 59.4 J/cm² and collected at 0, 3, 6, 12 and 24 hr post-PHT. The cells were trypsinized and washed twice in ice-cold PBS. The cells (1 × 10⁶) were then resuspended in 100 μl of Annexin V incubation reagent (TACS® Annexin V-FITC, Trevigen Inc.) which consisted of 10 μl 10X binding buffer, 10 μl PI, 1 μl FITC-conjugated Annexin V and 79 μl distilled water. The cells were then incubated in the dark for 15 min. Following this, 400 μl of 1X binding buffer was added to each tube, and cells were analyzed within 1 hr using a FACSAria cell sorter (BD Bioscience). The data were acquired with the FACSDiva 6.0 software program (BD Bioscience).

Caspase analysis by colorimetry: After PHT, the activity levels of caspase-8, caspase-9 and caspase-3 were determined by FLICE/caspase-8, FLICE/caspase-9 and FLICE/caspase-3 colorimetric assay kits (BioVision, Milpitas, CA, U.S.A.) as described in the manufacturer’s protocols. The intensity of the developed color was read at 405 nm in a microplate reader for 150 μg of total protein.

Western blotting: After PHT, cells were washed in PBS and lysed in RIPA buffer (25 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) with Halt protease inhibitor cocktail (Thermo Scientific, Rockford, IL, U.S.A.) on ice. Then the cells were scraped and cleared by centrifugation (15,000 g, 15 min, 4°C). The protein concentration was measured using a Bio-Rad protein assay kit. Equal amounts of protein (20 μg) were diluted in loading buffer (EZApply; Atto, Tokyo, Japan), boiled for 5 min and loaded on 10–12.5% sodium dodecyl sulfate – polyacrylamide gels for electrophoresis (SDS-PAGE; Atto, Tokyo, Japan), then transferred to a PVDF membrane. The blot was blocked with blocking buffer (5% nonfat dry milk in 1% Tween 20 in 20 mM Tris buffered saline) for 1 hr at room temperature, followed by immunoblotting with a primary antibody overnight at 4°C. The primary antibodies for Cyclin D1 (1: 500), Cyclin A (1:500) and Cyclin B (1:500) were from NeoMarkers (Fremont, CA, U.S.A.), antibodies against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1: 250) as an internal control, and heat shock protein (Hsp) 70/72 (1:1000) were from Enzo Life Sciences (Farmingdale, NY, U.S.A.). The secondary antibody was a goat anti-rabbit horseradish peroxidase (HRP)-conjugated (1:2000) antibody from Dako (Glostrup, Denmark). The Immobilon Western chemiluminescent HRP substrate (Millipore, Billerica, MA, U.S.A.) was added, and the signal was captured using a Chemi Doc XRS imaging system (Bio-Rad Laboratories, Hercules, CA).

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

RESULTS

PHT induces apoptosis in B16F10 cells: To investigate whether the effects of PHT on B16F10 growth inhibition were related to apoptosis, the cellular apoptosis was evaluated by flow cytometry by detection of Annexin V-FITC bound to externalized phosphatidylserine at the cell surface. Annexin V-FITC+/PI- staining defined cells in early apoptosis, Annexin V-FITC+/PI+ as those in late apoptosis, and Annexin V-FITC-/PI+ as those undergoing necrosis. As shown in Fig. 1A-F, the viable population (Q3) of cells gradually shifted to an apoptosis population (Q4). Together, these data suggest that PHT inhibits the proliferation of B16F10 cells via the induction of apoptosis, and that the induction of apoptosis seems to be very acute, with the maximum apoptotic cell population observed at 12 hr post-PHT.
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then decreased from 11 to 5% from 12 to 24 hr post-PHT. A significant increase in the number of both early and late apoptotic cells was observed compared with the control group (P<0.05). The percentage of early apoptosis was greater than that of late apoptosis (Fig. 1G). However, the activation of caspases 8, 9 and 3 was not detected from the colorimetric analysis (data not shown).

**PHT induces G0/G1 cell cycle arrest in B16F10 cells:** To further study the mechanism(s) by which PHT inhibits cell proliferation, the effects of PHT on the cell cycle distribution were evaluated by flow cytometry after propidium iodide staining. As seen in Fig. 2A, the percentage of cells in the sub-G1 phase in the control, 0, 3, 6, 12, and 24 hr groups were 1.5, 5.2, 9.2, 19.0, 39.1, and 47.0%, respectively. As shown in Fig. 2B, the percentages of G0/G1 phase cells in the control, ICG and light groups were between 39–40%. The percentage of G0/G1 phase cells in the 0 hr post-PHT group was significantly higher (P<0.05) than that of the control group, with 55% of the cells residing in G0/G1 phase. From 3 to 12 hr post-PHT, the percentage of cells in the G0/G1 phase was between 47–43%, which was higher but not significant than that of the control group. A concomitant decrease in the cell population in the S and G2/M phases was observed, but the differences between treatment groups were not significant.

Next, we performed a Western blot analysis to investigate the effects of PHT on the cell cycle regulation. PHT-mediated G0/G1 cell cycle arrest was confirmed by the downregulation of cyclin D1 expression in a time-dependent manner from 0, 3, and 6 to 12 hr post-PHT, as shown in Fig. 3. However, at 24 hr post-PHT, the cyclin D1 expression level was up-regulated. The expression levels of cyclin A and cyclin B were decreased over the designated time period by PHT (Fig. 3).

**The effects of PHT on the expression of Hsp70:** To study the effects of PHT on Hsp70 protein expression, a Western blotting analysis was performed. The Hsp70 protein expression was detected in the ICG group, and was up-regulated at 6, 12 and 24 hr post-PHT, but was not detected at 0 and 3 hr post-PHT (Fig. 3).

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**Fig. 1.** PHT induces apoptosis in B16F10 cells (A–F), Two color dot plot in quadrant: Q3 represents viable cell, Q4 represents early apoptotic cells, Q2 represents late apoptotic cells and Q1 represents necrotic cells. With increment of post PHT time, more cells from Q3 is moving towards Q4. (A) control, (B) 0 hr post-PHT. (C) 3 hr post-PHT. (D) 6 hr post-PHT. (E) 12 hr post-PHT. (F) 24 hr post-PHT. (G) Quantitative analysis of cell apoptosis with Annexin-V FITC and PI double staining. Data were mean ± SE (n=3). * and ** indicate a significance difference from control group, P<0.05.
In this study, we demonstrated the immediate induction of cell cycle arrest and apoptosis after PHT in B16F10 cells. We found that PHT can inhibit the proliferation of B16F10 cells in vitro at a concentration of 150 µM of IcG, and that this was associated with cell cycle arrest and apoptosis. A cell cycle analysis evaluating the DNA content demonstrated that the PHT-mediated cell growth inhibition was attributable, at least in part, to G0/G1 cell cycle arrest, and that this was detected immediately after treatment in this study. The G0/G1 cell cycle arrest was previously observed in McF-7 human breast cancer cell lines at 8 hr post-indocyanine green mediated PDT [3], and in SiHa human cervical carcinoma cells at 24 hr post-phthalocyanine-mediated PDT [15]. From these data, we concluded that PHT required a shorter time to exert its effects in vitro compared to PDT.

A cell cycle analysis evaluating the DNA content demonstrated that the PHT-mediated cell growth inhibition was attributable, at least in part, to G0/G1 cell cycle arrest, and that this was detected immediately after treatment in this study. The G0/G1 cell cycle arrest was previously observed in MCF-7 human breast cancer cell lines at 8 hr post-indocyanine green mediated PDT [3], and in SiHa human cervical carcinoma cells at 24 hr post-phthalocyanine-mediated PDT [15]. From these data, we concluded that PHT required a shorter time to exert its effects in vitro compared to PDT. Ormeod, et al. [30] studied the effects of hyperthermia on Chinese hamster V-79 cells, and found that although the cells stop synthesizing DNA after being heated at 43°C, the cells recover their normal cell cycle rate after 8–12 hr at 37°C. Furthermore, Flour, et al. [6] reported a non-significant increase in the number of G0/G1 arrested cells after the osteosarcoma cell line, ROS 17/2.8, was maintained at 40°C for 3 days. These reports suggest that HT alone is not effective for inducing cell cycle arrest.

Based on our study, we concluded that PHT was able to induce acute G0/G1 cell cycle arrest, and that arrested cells were able to be detected at 0 hr post-PHT (immediately after PHT) in B16F10 cells. Cell cycle progression is strictly regulated by a series of cyclins and cyclin-dependent kinases. Specific cyclin expression defines the relative position of the cell within the cell cycle [11]. The cyclin D1-associated kinase complex is responsible for G1 progression and is a key regulator of cell proliferation [27]. In our PHT study, the cyclin D1 protein expression was downregulated, which was in agreement with our observation of G0/G1 cell cycle arrest. However, at 24 hr post-PHT, cyclin D1 protein expression was detected, indicating that some cells that undergo G0/G1 cell cycle arrest can either proceed to apoptosis or regain the ability to enter the cell cycle. This phenomenon suggests that repeated PHT may be necessary to achieve good cell killing. PHT is capable of downregulating cyclin D1 expression (which is an oncogene) [44] and inhibited the proliferation of B16F10 melanoma cells in our study.
Cyclin A-associated kinase activities are needed for entry into the S phase, completion of the S phase and entry into the M phase [41]. The cyclin B-associated complex is needed for mitosis to occur [36]. Although no significant differences in the number of cells in the S and G2/M phases of the cell cycle were observed between the control and treatment groups by the flow cytometric analysis, the protein expression study showed that cyclin A and cyclin B were degraded in the PHT groups, indicating that PHT is able to affect B16F10 proliferation in any phase of the cell cycle.

We found that PHT is able to induce acute and persistent apoptosis in B16F10 cells and earlier apoptosis compared to PDT and HT. A few studies have demonstrated apoptosis post-PDT, but the time required for apoptosis or photosensitization were longer than in the present study [24, 25]. The HT treatment duration required to induce apoptosis also ranged from 30 to 90 min [37, 43]. In the present study, even at 0 hr post-PHT, evidence of apoptosis in the sub-G1 population in the cell cycle analysis and phosphatidylserine (PS) externalization (an indicator of early apoptosis) were detected. Phosphatidylserine primarily located on the inner surface of lipid bilayer of plasma membrane facing the cytosol. These findings suggested that ICG with photodynamic therapy, combined with heat to exert acute and persistent (up to 24 hr) apoptosis. The possible mechanism of acute apoptosis in this study can be due to the synergistic effects of PDT and HT. The photo-oxidative damage caused by singlet oxygen produced by PDT with ICG is amplified by the free radicals generations in the membrane or cytoplasm of tumor cells by HT leading to lipid peroxidation [23, 48]. Protein oxidation impairs the redox equilibrium needed for proper function of many metal-containing enzymes including glutathione peroxidase, and catalases, which in turn reduces the cellular ability to eliminate reactive oxygen species [32]. This intensified oxidative stress creates massive imbalance between pro-oxidant and anti-oxidant systems, thus accentuate the cytotoxic effect [8].

Apoptosis can be triggered by both intrinsic and extrinsic pathways [19]. Generally, the PDT-mediated apoptotic pathways involve the activation of a cascade of caspases 8, 9, and 3. However, we did not detect increased activity of caspases 8, 9, and 3 using a colorimetric analysis. Hersey [16] reported that melanoma was resistant to extrinsic and/or intrinsic apoptotic pathways due to activation of the Ras/Raf/MEK/ERK pathways and phosphatidylinositol 3-kinase/Akt pathways. Growth factors and mitogens use the Ras/Raf/MEK/ERK signaling cascade to transmit signals from their receptors to regulate gene expression and prevent apoptosis [26]. The activation of Akt has been implicated in the progression of melanoma by inducing apoptosis resistance in melanoma cells [4, 40].

PHT might induce cell death via a caspase-independent pathway. Upon loss of mitochondrial membrane integrity, apoptosis-inducing factor (AIF), which is located between the inner and outer mitochondrial membranes, is released and translocated into the nucleus to induce nuclear condensation [2]. Vittar et al. [46] reported that phthalocyanine causes caspase-independent apoptosis in MCF-7c3 breast cancer cells after PDT. In addition, hyperthermia can...
induce endoplasmic reticulum-mediated apoptosis [39]. Hence, the PHT used in this study might elicit apoptotic death via a caspase-independent pathway involving endoplasmic reticulum-mediated apoptosis. Further studies are needed to clarify the PHT-related cell death pathways.

Hsp70 has strong cytoprotective properties and its overexpression prevents cell death triggered by various stimuli, including hyperthermia and oxidative stress [10]. In this study, Hsp70 was upregulated from 6 hr post-PHT onward, indicating that there was a delay in Hsp70 protein expression, giving more time for apoptosis and necrosis to occur. Fournier et al. [7] had observed a temporal delay in the correlation of the mRNA and protein expression, where the mRNA expression at 1 and 2 hr correlated best with the protein expression changes after 6 hr of rapamycin treatment. This allowed for the induction of heat shock and the inhibition of ribosome biogenesis. Hsp70 expression may be the reason why the G0/G1 arrested B16F10 cells were able to resume cell cycle progression again, as indicated by the upregulation of cyclin D1. Hsp70 can inhibit apoptosis by neutralizing and interacting with apoptosis-inducing factor (AIF) translocated into the nucleus [13]. Therefore, our present data suggest that PHT rapidly elicits apoptosis before Hsp70 protein expression is induced, but that it is not able to prevent Hsp70 formation.

In summary, we showed that PHT elicits rapid and persistent apoptosis with G0/G1 cell cycle arrest compared to PDT and HT alone in B16F10 melanoma cells. The cell cycle arrest and apoptosis occurred as interrelated events in response to PHT. Repetitive treatments with PHT may be necessary to induce apoptosis in all of the target cells. Further studies are needed to clarify the mechanism(s) responsible for the apoptosis resulting from PHT, to relate the Hsp70 expression level with apoptosis and PHT effects on other cancer cell lines.

REFERENCES

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