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The Effect of Photodynamic Therapy on Tumor Cell Expression of Major Histocompatibility Complex (MHC) Class I and MHC Class I-Related Molecules

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Abstract

Background and Objective—Photodynamic therapy (PDT) is FDA-approved anti-cancer modality for elimination of early disease and palliation in advanced disease. PDT efficacy depends in part on elicitation of a tumor-specific immune response that is dependent on cytotoxic T lymphocytes (CTLs) and natural killer (NK) cells. The cytolytic potential of CTLs and NK cells is mediated by the ability of these cells to recognize major histocompatibility complex (MHC) class I and MHC class I-related molecules. The MHC class I-related molecules MICA and MICB are induced by oxidative stress and have been reported to activate NK cells and co-stimulate $CD8^+$ T cells. The purpose of this study was to examine the effect of PDT on tumor cell expression of MHC classes I and II-related molecules in vivo and in vitro.

Study Design/Materials and Methods—Human colon carcinoma Colo205 cells and murine CT26 tumors were treated with 2-[1-hexyloxyethyl]-2-devinyl pyropheophor-bide-a (HPPH)-PDT at various doses. MHC classes I and I-related molecule expression following treatment of Colo205 cells was temporally examined by flow cytometry using antibodies specific for components of MHC class I molecules and by quantitative PCR using specific primers. Expression of MHC class I-related molecules following HPPH-based PDT (HPPH-PDT) of murine tumors was monitored using a chimeric NKG2D receptor.

Results—In vitro HPPH-PDT significantly induces MICA in Colo205 cells, but had no effect on MHC class I molecule expression. PDT also induced expression of NKG2D ligands (NKG2DL) following in vivo HPPH-PDT of a murine tumor. Induction of MICA corresponded to increased NK killing of PDT-treated tumor cells.

Conclusions—PDT induction of MICA on human tumor cells and increased expression of NKG2DL by murine tumors following PDT may play a role in PDT induction of anti-tumor

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immunity. This conclusion is supported by our results demonstrating that tumor cells have increased sensitivity to NK cell lysis following PDT.

Keywords

PDT; NKG2D; MICA; MHC class I

INTRODUCTION

Photodynamic therapy (PDT) causes tumor destruction through the generation of reactive oxygen species (ROS), vascular disruption, induction of inflammation, and enhancement of anti-tumor immunity [1]. ROS are produced upon illumination of tumors with a specific wavelength of light following administration of a photoreactive drug or photosensitizer. PDT is approved for clinical use in a number of countries, including the United States, for the elimination of early stage malignancies, palliation of symptoms, and reduction of obstruction in patients with late stage tumors [2,3].

PDT was initially considered to be primarily a local treatment; however, several studies have shown that local PDT treatment can lead to the induction of systemic anti-tumor immunity, resistance to subsequent tumor challenge [4,5], and an ability to combat distant disease [6–11]. Induction of anti-tumor immunity following PDT is dependent upon the presence of $CD8^+$ T cells [4,5] and in some instances, natural killer (NK) cells [6]. While a majority of the studies demonstrating enhancement of anti-tumor immunity by PDT have been done in pre-clinical models by Abdel-Hady et al. [12] demonstrated that PDT outcome in patients can be linked to $CD8^+$ T-cell infiltration. In addition, Kabingu et al. [13] showed that PDT of basal cell carcinoma (BCC) leads to enhanced immune recognition of a peptide derived from the BCC-associated antigen, HIP-1, when the peptide is presented in the context of the major histocompatibility complex (MHC) class I molecule HLA-A2.

Effector $CD8^+$ T lymphocytes (CTLs) recognize target cells via engagement of T-cell receptor (TcR) with peptide-bearing classical MHC class I molecules on the surface of target cells. TcR:peptide:MHC class I binding results in activation of CTLs and killing of target cells [14]. NK cells express MHC class I-specific receptors that when engaged can inhibit NK cell killing of target cells [15]. Thus, recognition of tumor cells by both $CD8^+$ T and NK cells depends upon expression of classical MHC class I molecules.

In addition to classical MHC class I molecules, both $CD8^+$ T cells and NK cells are activated by recognition of MHC class I-like proteins on target cells [16]. These molecules are recognized by the NKG2D receptor and are globally denoted as NKG2D ligands (NKG2DL) [17,18]. Human NKG2DL include MHC-class I polypeptide-related chain (MIC) A and B and the cytomegalovirus UL16-binding proteins (ULBP) 1–4 and REATIG. Mice do not express MIC or ULBP proteins; ligands of murine NKG2D include the five retinoic acid early transcript 1 (RAE1) proteins, H60 and MULT-1.

NKG2DL recognition has been shown to play a major role in immune recognition and elimination of tumors [16,19–22]. NKG2DL expression is induced by many insults, including oxidative stress [23]. The dependence of PDT on $CD8^+$ T cells and NK cells as well as the induction of NKG2DL by oxidative stress prompted us to examine the effect of 2-[1-hexyloxyethyl]-2-devinyl pyropheophor-bide-a (HPPH)-based PDT (HPPH-PDT) on the expression of classical and non-classical MHC class I molecules. HPPH is a second generation photosensitizer that was developed to overcome the side effects associated with the first generation sensitizer, sodium Porfimer [24]. HPPH-PDT has shown excellent

efficacy in both pre-clinical and clinical studies [24,25] and was chosen for this research based on its ability to stimulate anti-tumor immunity [26].

We demonstrate that HPPH-PDT of colon carcinoma cells of human and murine origin results in increased expression of the NKG2DL, MICA. This increase in MICA expression is correlated with an increase in NK cytotoxicity against PDT-treated tumor cells.

MATERIALS AND METHODS

Cell Lines and Reagents

The human colon carcinoma cell line Colo205, murine colon carcinoma cell line CT26, and human NK cell line NK-92MI were purchased from ATCC (Manassas, VA). Colon carcinoma cell lines were grown in RPMI-1640 medium purchased from Life Technologies (Grand Island, NY), supplemented with 10% bovine calf serum purchased from Hyclone Laboratories (Logan, UT), and 10 mM HEPES. NK-92MI is a subclone of NK-92, which is an IL-2-dependent NK cell line derived from a patient with non-Hodgkin's lymphoma. NK-92MI was generated by transfection of the parental line with human IL-2 cDNA [27] and grows independent of IL-2. NK-92MI were propagated in alpha minimum essential medium supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate, 0.2 mM inositol, 0.1 mM 2-mercaptoethanol; 0.02 mM folic acid, 12.5% horse serum, and 12.5% fetal bovine serum. All cells were grown in a Heraeus incubator (Fisher Scientific, Pittsburgh, PA) at 37°C and 5% CO_2.

The transporter associated with antigen processing (TAP1)-specific antibody NOB-1, TAP-associated protein (tapasin)-specific antibody TO-3, and the MHC class I heavy chain-specific antibody TP25.99 have been previously described [28]. The mAb specific for human tubulin was purchased from Sigma–Aldrich (St. Louis, MO). Antibodies specific for STAT3 and the pan-specific antibodies for human classical MHC class I molecules were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). MICA antibody, Amo-1, and MICB antibody BMO-2 were purchased from Axxora (San Diego, CA). Antibodies to ULBPs and the murine NKG2D-Fc were purchased from R&D Systems (Minneapolis, MN). The CD45-specific antibody was purchased from BD Biosciences (Bedford, MA).

Animals

BALB/cJ mice purchased from Jackson Labs (Bar Harbour, ME) and housed in the Roswell Park Cancer Institute Animal Facility. Following tumor inoculation, animals were monitored daily. The Roswell Park Cancer Institute Institutional Animal Care and Use Committee (IACUC) approved all procedures carried out in this study.

Photodynamic Therapy

The clinical grade HPPH was obtained from the Roswell Park Pharmacy and reconstituted to 0.4 mmol/L in pyrogen-free 5% dextrose in water (D5W; Baxter Corp). Unless otherwise specified, for in vitro treatment with PDT, cells were incubated at 37°C and 5% CO_2 with HPPH for 21 hours in the dark. Following the 21-hour incubation the excess photosensitizer was removed by a single wash in PBS. Cells were then incubated under the same conditions for an additional 3 hours. After the 3-hour incubation in the dark, cells were illuminated with 665 nm light given at 600–630 mW/cm^2 using an argon laser-pumped dye laser (Spectra Physics, Mountain View, CA).

For in vivo PDT, animals were inoculated subcutaneously (s.c.) on their left shoulder with 3 × 10^5 CT26 a murine colon carcinoma cell line and allowed to grow for approximately 6 days or until they reached a size of 5 mm × 5 mm. Tumor growth was monitored every other

day using HPPH was injected into the tail vein at a dose of 0.4 μmoles/kg. After 18–24 hours, the primary s.c. tumors were exposed to 665 nm light until a total fluence of 100 J/cm^2 was achieved at a fluence rate of 75 mW/cm^2. Animals treated with HPPH alone, were used as controls.

MTT Assay

An aliquot of cells was removed after the 21-hour incubation in photosensitizer or media (control cells) and plated in multiple wells of a 96-well plate at various concentrations. Plated cells that had not been incubated with the photosensitizer were incubated at 37°C and 5% CO_2 for 3 hours and then either treated with light or not (light only control and untreated control, respectively) and so were the plates containing the cells incubated with HPPH (HPPH-PDT-treated and HPPH only control, respectively). Twenty-four hours after the treatment with PDT, 10 μl of a 4 mg/ml stock of MTT (Sigma–Aldrich) was added to all the wells and incubated in the dark for 3 hours. Following the 3-hour incubation during which the viable cells metabolized the MTT reagent to generate purple crystals, all the plates cells were spun down to preserve semi-adherent cells to the bottom of the plate and the MTT excess present in the media was removed. DMSO (100 μl) was added to all the wells and the plates were rocked at room temperature in the dark for 30 minutes. The optical density (OD) at 560 nm was determined. The photosensitizer only control was set as background given the dark toxicity observed at the HPPH concentration used during our studies.

Western Blotting

Quantitative fluorescent-based Western blotting was performed as described previously [29]. Briefly, cells were lysed using 1% Triton X-100 in 10 mM Tris, 150 mM NaCl, pH 7.4 supplemented with protease inhibitors PMSF (0.5 mM), and *N*-ethylmaleimide (5 mM), and incubated at 4°C for 30 minutes. Post-nuclear protein concentrations were determined by the Bradford assay (Bio Rad, Hercules, CA) and separated by SDS–PAGE. Following transfer of proteins to Immobilon-P membrane (Millipore, Bedford, MA), membranes were incubated in 3% bovine serum albumin (BSA) to reduce background signal. Proteins of interest were detected using the indicated primary antibodies (incubated overnight in an orbital shaker at 4°C). Primary antibodies were decanted and membranes were washed a minimum of three times in PBS/0.02% Tween 20. Secondary antibodies coupled to alkaline phosphatase were then added in 3% BSA and incubated at room temperature for 1 hour. After three washes in PBS/0.02% Tween 20, VistraECF substrate (Amersham Pharmacia, Piscataway, NJ) was applied and fluorescence was measured using a STORM instrument (Molecular Dynamics, Palo Alto, CA). Fluorescence measurements were acquired and analyzed using ImageQuant software.

Flow Cytometric Staining

Cells were washed and incubated for 60 minutes at 4°C with saturating concentrations of the indicated primary antibodies in FACS buffer. After washing three times to remove the excess primary antibody, 5×10^5 cells were resuspended in FACS buffer in saturating concentrations (10 μg/ml) of fluorescein isothiocyanate (FITC) or phycoerythrin (PE) conjugated, goat anti-mouse IgG antibodies for 30 minutes at 4°C in the dark. Cells were then washed three times to remove the excess secondary antibody and fixed in 2% paraformaldehyde. Isotype and experimental samples were analyzed on a FACScan instrument (Becton Dickinson, San Jose, CA). Data were analyzed with WINMDI software (Joe Trotter, The Scripps Research Institute, La Jolla, CA) or FlowJo (Ashland, OR).

RNA Extraction, RT-PCR, Q-PCR

RNA from cells was isolated using TRIZOL (Invitrogen, Carlsbad, CA) as per the manufacturer's recommendations. Two micrograms of RNA was reverse transcribed to generate cDNA using the Superscript II system (Invitrogen) and genes of interest were amplified using Accuprime taq (Invitrogen). For quantitative-PCR (Q-PCR), 2 μl of the cDNA reaction was used to amplify the indicated genes with the aid of SYBR green technology. Samples were set in triplicate wells and normalized to GAPDH and β-actin. The master mix used for the Q-PCR was purchased from Promega (Madison, WI) and the reactions were performed by Applied Biosystems HT7900 machine (Foster City, CA). The primers were designed at the 3′-end of the gene and always spanning an intron to easily detect genomic contamination if at all present. The 5′ to 3′ sequence of the primer sets were: β2-microglobulin (FP: GGCTATCCAGCGTACTCCAAA and RP: CGGCAGGCATACTCATCTTTTT), tapasin (FP: TATGCCTGTCGAATTCACCATCC and RP: TATGCCTGTCGAATTCACCATCC), heavy chain (FP: GATTACATCGCCTTGAACGAGG and RP: AGAGACAGCGTGGTGAGTCAT), GAPDH (FP: GAAGGTGAAGGTCGGAGTC and RP: GAAGATGGTGATGGGATTTC), β-actin (FP: TCCTGTGGCATCCACGAAA and RP: GCTGATCCACATCTGCTGGAA). Following either RT-PCR or Q-PCR, the amplicons size was confirmed by running the products in a 1.5–2.0% aga-rose gel in TAE. The sequence of the primer sets used to determine the induction of the indicated stress inducible genes were for MICA (FP: CCTTGGCCATGAACGTCAGG and RP: CGCAGCGAGGCCTCAGAGG), MICB (FP: ACC TTGGCTATGAACGTCACA and RP: CCCTCTGAGACCTCGCTGCA), ULBP-1 (FP: GTACTGGGAACAAATGCTGGAT and RP:AACTCTCCTCATCTGCCAGCT), ULBP-2 (FP: TTACTTCTCAATGGGAGACTGT and RP: TGTGCCTGAGGACATGGCGA), and ULBP-3 (FP: CCTGATGCACAGGAAGAAGAG and RP: TATGGCTTTGGGTTGAGCTAAG). All primer sets were purchased from Integrated DNA Technologies (San Diego, CA).

NK Cytotoxicity Assay

NK cytotoxicity was measured using a flow cytometry-based assay as previously described [30]. Colo205 cells were treated with HPPH-PDT (0.3 J/cm^2) and incubated overnight at 37°C, 5% CO_2. Following incubation, PDT-treated cells were washed once and labeled with a cyanine membrane dye 3,3′-dioctadecyloxacarbocyanine (DiO, 30 μM) by a 20-minute incubation at 37°C. NK92MI cells and the target cell lines were then incubated for 2 hours at 37°C in the presence of 75 μM propidium iodide (PI; Molecular Probes, Eugene, OR). After the incubation, the samples were analyzed by flow cytometry. A minimum of 2,000 target cells was analyzed per sample and dead target cells were identified as DiO^+PI^+. Results are presented as percent-specific lysis which was calculated as follows: percent-specific lysis = [(observed experimental lysis – the spontaneous lysis observed when target cells were incubated with medium alone)/(maximal lysis observed when target cells alone were incubated with 0.3% Triton X-100 – the spontaneous lysis)] × 100.

Statistical Analysis

Statistical analyses were performed using a standardized Student's *t*-test with Welch's correction, where equal variances were not assumed, to compare experimental groups. Differences were considered significant when *P*-values were ≤0.05.

RESULTS

Determination of PDT Efficacy on Colo205 Cells

Colo205 cells were subjected to HPPH-PDT to establish a dose–response curve. A summary of the in vitro treatment strategy can be found in Figure 1A; Colo205 cells incubated with HPPH at 0.8 μM were exposed to increasing fluences (0.05–1 J/cm^2 administered at 600–630 mW/cm^2) and their viability was determined 24 hours post-treatment by MTT assay (Fig. 1B). Subsequent studies were performed at LD30 (0.2–0.3 J/cm^2) or over a dose range of LD30–LD99 (>1 J/cm^2).

Previous studies have shown that the signal transducer and activator of transcription 3 (STAT-3) is cross-linked following PDT treatment and that the magnitude of the cross-linking directly correlates with the combination of the photosensitizer and fluence [31]. To confirm the response to HPPH-PDT seen in Colo205 cells, the amount of STAT-3 cross-linking was determined by Western blot (Fig. 1C). As expected, the amount of cross-linked STAT-3 increased with treatments of higher fluence.

PDT Does Not Affect the Regulation, Protein Level or Surface Expression of Classical MHC Class I Molecules

Long-term control of PDT-treated tumors relies on CD8$^+$ T cells and NK cells [4,5,32]. CD8$^+$ T cells recognize antigens by ligation of TcR to MHC class I molecules, which are composed of MHC class I heavy chain, β2-microglobulin, and cognate peptide. It has been shown in several models that expression levels of MHC class I surface molecules regulates the CD8$^+$ T cell and NK cell response to tumors [15,33]. To evaluate whether HPPH-PDT modulates the expression of classical MHC class I molecules, we examined the mRNA induction of MHC class I heavy chain and β2-microglobulin by Q-PCR on control (HPPH alone) and HPPH-PDT-treated Colo205 cells. RNA was isolated at different time points following treatment at 0.3 J/cm^2 (LD30) to determine the effect on the indicated genes. Samples were analyzed by Q-PCR using the $\Delta\Delta C_t$ method and normalized to housekeeping genes GAPDH and β-actin. Q-PCR analysis revealed the lack of an induction of the examined genes immediately, 1, 3, 6, 12, or 24 hours following treatment (Fig. 2A).

Surface expression of classical MHC class I molecules is controlled by several proteins in the endoplasmic reticulum (ER) [34]. Among the many proteins involved in assembly and loading of classical MHC class I molecules, the TAP and tapasin have been shown to be necessary for maximal expression at the cell surface [35,36]. Control or HPPH-PDT-treated (0.3 J/cm^2) Colo205 cells were isolated 1, 6, 12, or 24 hours post-treatment and analyzed for the indicated proteins by Western blot. Analysis of total cell lysates revealed that HPPH-PDT did not affect the protein level of any of the tested proteins (Fig. 2B).

To rule out the possibility that the distribution of MHC class I molecules and not their total level is altered, cell surface expression of classical MHC class I molecules was determined pre-PDT and 24 hours post-PDT as a function of fluence. Cell surface expression of MHC class I molecules was not altered 24 hours after post-PDT at any of the tested fluences (Fig. 2C). Expression was also unchanged when cells treated with LD60 dose of HPPH-PDT were examined 48 hours post-treatment (Fig. 2D). In total these results suggest that factors other than MHC class I expression are responsible for increased NK and CD8$^+$ T-cell cytolytic activity following PDT.

The NKG2DL MICA Is Induced Following PDT

To examine the effect of HPPH-PDT on NKG2DL expression, Colo205 cells were treated with HPPH alone or HPPH-PDT (0.3 J/cm^2; LD30). Cells were examined by flow

cytometric analysis using a MICA-specific antibody 24 hours following treatment. HPPH-PDT significantly enhanced MICA expression at the cell surface when compared to untreated or HPPH-treated cells (Fig. 3A). HPPH treatment alone also significantly increased MICA expression over that observed in untreated cells. The effect of HPPH-PDT on MICB was determined by RT-PCR. MICB mRNA is expressed constitutively by Colo205 cells and was unaltered by HPPH-PDT (Fig. 3B). The gene promoters of the NKG2DL contain heat shock responsive elements [18,23]; therefore hyperthermia was used as a positive control for gene expression. MICA expression was induced by treatment of Colo205 cells with hyperthermia (Fig. 3D); hyperthermia had no effect on MICB expression (data not shown).

Treatment of Colo205 cells with HPPH-PDT failed to induce cell surface expression of ULBP family members at any fluence tested (Fig. 3C). ULBP-2 and -3 expression was increased by treatment with hyperthermia (Fig. 3D), indicating that the lack of induction of ULBP family members was not due to an inability of Colo205 cells to express these ligands.

MICA expression can also be increased on colon carcinoma cells by hydrogen peroxide (H_2O_2) treatment [37]; therefore PDT induction of MICA on Colo205 cells was also compared to induction by H_2O_2. Colo205 cells were treated with HPPH-PDT at 0.3 J/cm^2 or H_2O_2 at 50 and 250 μM. MICA surface expression was determined 24 hours post-treatment by flow cytometric analysis. H_2O_2 treatment resulted in a significantly higher induction of MICA when compared to HPPH-PDT (Fig. 4).

PDT Results in the Induction of NKG2D Ligands In Vivo

To determine if our findings translate to the in vivo setting, CT26 tumor-bearing animals were treated with HPPH-PDT at a tumor LD50 and the surviving cells were examined for the expression of MHC class I and NKG2DL expression. PDT-treated and control tumors were harvested 24 hours post-treatment and a single cell suspension was generated [38]. Tumor cells were distinguished from host immune cells as those cells lacking CD45. A chimeric NKG2D receptor moiety was used to detect NKG2DL expression by flow cytometry. NKG2DL expression was increased on tumor cells following HPPH-PDT treatment of CT26 tumors (Fig. 5); MHC class I expression was unchanged by PDT (data not shown).

PDT Treatment Enhances NK Cytotoxicity

Previous studies have shown that increased expression of NKG2DL leads to enhanced NK cytotoxicity [16,19–22,39]. Therefore, we tested whether PDT-treated Colo205 cells showed increased sensitivity to NK lysis. Colo205 cells were treated with HPPH-PDT (0.3 J/cm^2) or H_2O_2 (50 μM) and incubated for 24 hours to allow expression of MICA. Treated cells were then cultured in the presence of a human NK cell line (NK-92MI) and specific cytotoxicity was determined by flow cytometric analysis. Treatment with either HPPH-PDT or H_2O_2 significantly increased tumor cell susceptibility to NK lysis at effector to target ratios of 5:1 and 10:1 (P 0.01 when compared to cells treated with PBS alone); H_2O_2-treated target cells were also significantly more sensitive to lysis by NK-92MI cells at ratios of 25:1 and 50:1. These findings correspond to the degree of MICA induction found following treatment with HPPH-PDT and H_2O_2 (Fig. 6).

DISCUSSION

We show that PDT of human colon carcinoma cells in vitro and murine colon tumors in vivo leads to increased expression of non-classical MHC class I molecules. These molecules are recognized by the cytotoxicity receptor NKG2D expressed on CD8$^+$ T cells and NK cells.

The induction of NKG2DL expression in human Colo205 colon carcinoma cells by PDT appears to be limited to induction of MICA. We were unable to detect induction of either MICB or any of the ULBP family members. In contrast to the induction of MICA by PDT, treatment does not result in increased expression of either classical MHC class I molecules or molecules associated with antigen presentation by MHC class I molecules. Induction of MICA by HPPH-PDT corresponded to an increase in NK killing of the PDT-treated tumor cells.

NKG2DL expression can be induced by oxidative stress [23,40]. Interestingly, induction of MICA by PDT was significantly less than that observed following treatment with H_2O_2. It is possible that the half-life of PDT-induced ROS is too short to allow for gene induction or that PDT induction of MICA is independent of oxidative stress. This possibility is supported by the results showing that treatment with HPPH alone leads to induction of MICA. HPPH localizes to the mitochondrial membrane [41]; mitochondrial damage or stress can lead to NF-κB activation and cytokine secretion [42], which may lead to increases in MICA expression [16,23].

The differential expression of MICA and MICB in response to HPPH-PDT is somewhat surprising given that these two molecules share significant homology and are encoded by evolutionarily conserved genes [43]. However, MICA and MICB are highly polymorphic and several studies have shown that they can be differentially regulated [23,40]. It is also apparent that NKG2DL expression and regulation varies between tumor cell lines [16]. Critical to our study are the findings showing that transcriptional regulation of NKG2DL is both cell type and environment dependent [16]. Importantly, previous studies have shown that hematoporphyrin-based PDT induces ULBP-1 and -2 genes in a gastric tumor cell line and MICA/B, ULBP-1, -2, and -3 in a lung cancer cell line [39].

Other studies have also examined the effects of PDT on MHC class I expression with mixed results. Verteporfin-based PDT lead to a downregulation of classical MHC class I surface expression on murine dendritic cells but had no effect on B cell expression of these molecules [44]. Blom et al. [45] demonstrated that hematoporphyrin-based PDT of human ocular melanoma cells led to rapid changes in expression that returned to pre-treatment levels within 6 hours. Unfortunately, our study does little to clarify these discrepancies other to further suggest that tumor cell expression of MHC class I appears to be unaffected by PDT. This conclusion is supported by the study of Abdel-Hady et al. [12], which shows that 5-aminolevulinic acid-based PDT is unable to induce MHC class I expression on tumor cells lacking MHC class I.

Our laboratory has previously shown that PDT enhanced anti-tumor immunity is dependent on $CD8^+$ T cells and NK cells. In the current study, we show that both in vitro treatment of human tumor cells and in vivo treatment of murine tumors with HPPH-PDT results in increased NKG2DL expression. Our studies and those of Park et al. [39] showed that PDT-induced NKG2DL expression was accompanied by increased sensitivity to NK cell killing. Therefore, it is likely that induction of NKG2DL following PDT contributes to PDT enhancement of anti-tumor immunity. Understanding the regulation of NKG2D by different types of PDT treatments may provide answers to the differing ability of various PDT photosensitizers to enhance anti-tumor immunity. Furthermore, this understanding is fundamental for the improvement and design of new treatment strategies that will elicit the long-term immune response mediated by PDT in the clinic.

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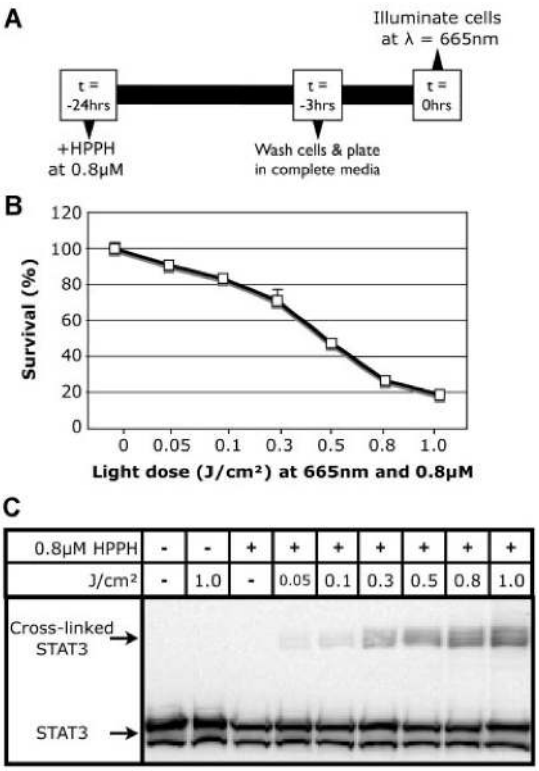

Fig. 1.

Determination of HPPH-PDT dose–response in Colo205 cells. **A**: Strategy for the in vitro treatment of Colo205 cells with HPPH-PDT. **B**: Colo205 cells were treated at several fluences and 24 hours post-HPPH-PDT their viability was determined by MTT. **C**: Colo205 cells were harvested immediately following treatment with HPPH-PDT at different fluences, lysed, and examined for the presence of STAT-3 cross-linking by Western blot.

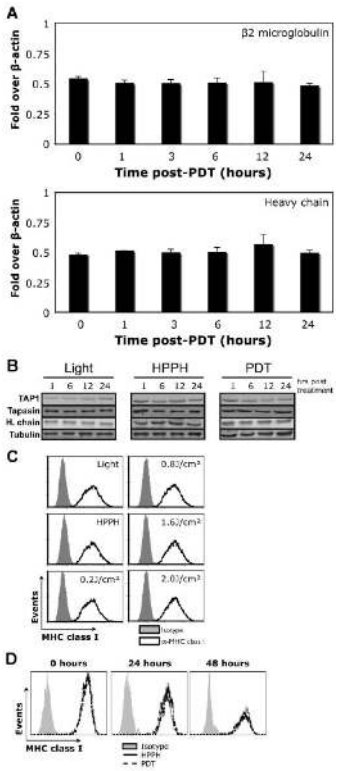

Fig. 2.

HPPH-PDT does not affect classical MHC class I molecule expression. **A**: Colo205 cells treated with HPPH-PDT at 0.3 J/cm^2 were harvested immediately following, or 1, 3, 6, 12, and 24 hours post-treatment. cDNA generated from each of the time points was used in Q-PCR reactions using primers specific to β2-microglobulin (**upper panel**) or MHC class I heavy chain (**lower panel**). Induction was normalized to both GAPDH and β-actin. **B**: Total protein levels for the indicated proteins were examined 1, 6, 12, and 24 hours post-HPPH-PDT at 0.3 J/cm^2. **C**: The surface expression of classical MHC class I molecules was determined as a function of fluence 24 hours post-HPPH-PDT. **D**: Surface levels of classical MHC class I molecules were examined as a function of time following HPPH-PDT at 0.8 J/cm^2.

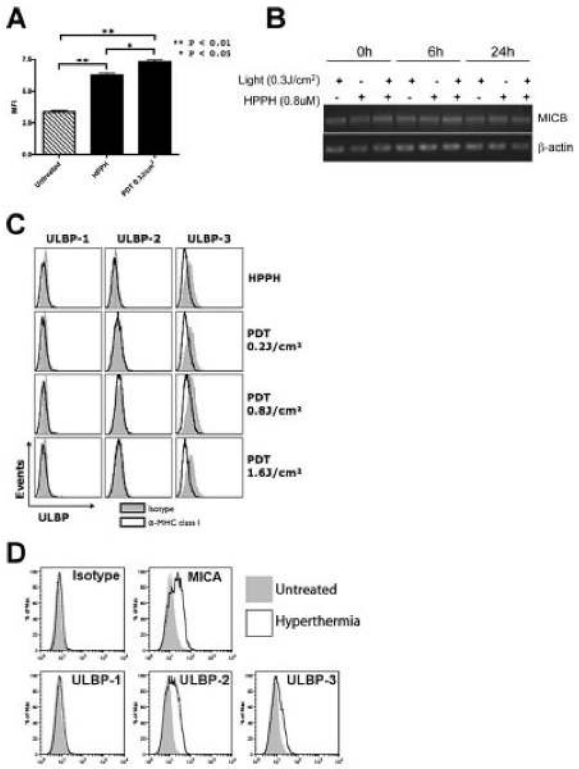

Fig. 3.

The human NKG2D ligand MICA is induced following HPPH-PDT. **A**: Untreated Colo205 cells, HPPH only or treated with HPPH-PDT at 0.3 J/cm^2 were examined 24 hours after treatment by flow cytometry for the expression of MICA at the cell surface. **B**: Changes in MICB mRNA levels were determined following HPPH-PDT 24 hours post-treatment. **C**: Colo205 cells treated with HPPH-PDT at 0.2, 0.8, and 1.6 J/cm^2 were analyzed for the expression of ULBP family members at the cell surface by flow cytometry 24 hours following treatment. **D**: Colo205 cells were either left untreated or treated with hyperthermia, 6-hour incubation at 39.5°C followed by a 16-hour rest period prior analysis with the indicated antibodies.

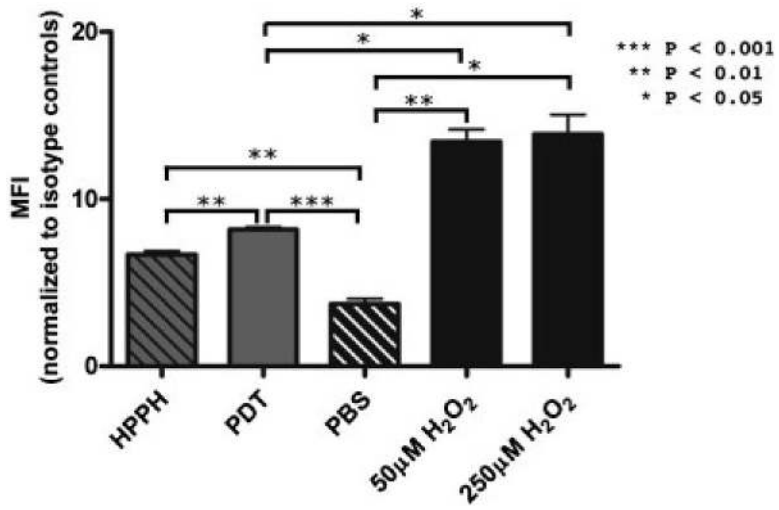

Fig. 4.

Comparison of MICA induction by HPPH-PDT and hydrogen peroxide. Colo205 cells treated with HPPH only or HPPH-PDT at 0.3 J/cm^2 were examined 24 hours after treatment by flow cytometry for the expression of MICA at the cell surface. MICA levels were also examined in cells incubated with hydrogen peroxide. Results are shown as average mean fluorescence intensity (MFI); error bars represent SEM.

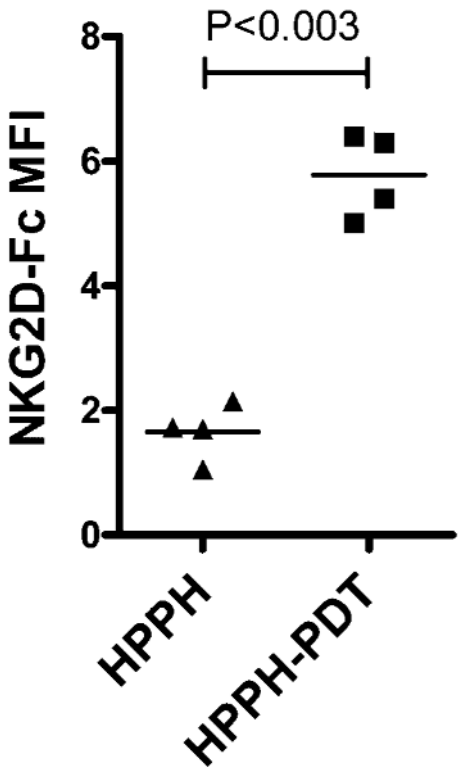

Fig. 5.

In vivo HPPH-PDT results in the significant induction of murine NKG2D ligands. BALB/c mice were inoculated with CT26 tumor cells in the left shoulder. Once the tumor reached a size of 5 × 5 mm^2, mice were injected with HPPH, and 24 hours later half of the animals were illuminated with light of a specific wavelength, while the other were used as controls (HPPH alone). Tumor tissue was isolated 24 hours post-PDT and a single cell suspension was generated. Tumor cells ($CD45^-$) were analyzed by flow cytometry for the expression of NKG2D ligands using an NKG2D-Fc chimera. Results are presented as NKG2D-Fc MFI; each symbol represents an individual tumor.

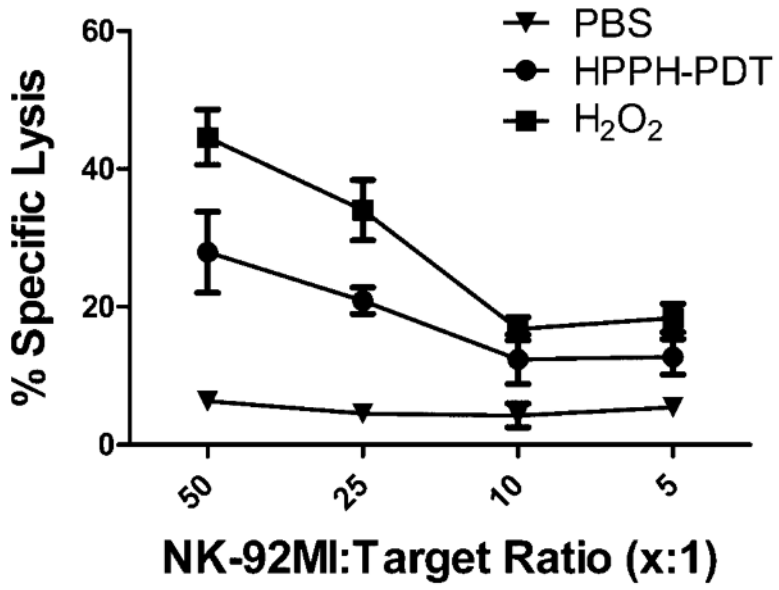

Fig. 6.

HPPH-PDT enhances NK killing of Colo205 cells. Colo205 cells were treated with PBS, HPPH-PDT (0.3 J/cm^2) or H_2O_2 (50 μM). Following a 24-hour incubation cells were used as target cells in a NK cytotoxicity assay as described in Materials and Methods Section. Results are shown as mean-specific lysis ± SEM.