Survivin, a Member of the Inhibitor of Apoptosis Family, Is Induced by Photodynamic Therapy and Is a Target for Improving Treatment Response

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Abstract

We observed that photodynamic therapy (PDT) induces the expression and phosphorylation of the inhibitor of apoptosis (IAP) protein survivin in murine and human cancer cells and tumors. Survivin inhibits caspase-9, blocks apoptosis, and is associated with resistance to chemotherapy and radiation. Survivin is a client protein for the 90-kDa heat shock protein (Hsp-90), and the binding of survivin to Hsp-90 assists in the maturation, proper folding, assembly, and transport of this IAP protein. A derivative of the antibiotic geldanamycin, 17-allylamino-17-demethoxygeldanamycin (17-AAG), interferes with proper binding of client proteins, such as survivin, to Hsp-90 and leads to misfolding of client proteins, ubiquitination, and proteasome degradation. We hypothesized that PDT efficacy may be reduced by treatment-mediated expression and phosphorylation of survivin, and therefore, targeting the survivin pathway could increase PDT responsiveness. To address this hypothesis, we examined cellular and molecular responses following exposure to PDT, 17-AAG, and the combination of PDT plus 17-AAG in human BT-474 breast cancer cells using Photofrin and NPe6 as photosensitizers. Cells treated with the combination of PDT and 17-AAG exhibited decreased expression of the Hsp-90 client proteins phosphorylated survivin, phosphorylated Akt, and Bel-2. The decreased expression of these client proteins was accompanied by higher apoptotic indexes and increased cytotoxicity. To confirm a specific role for survivin in modulating PDT, we used a human melanoma cell line, YUSAC2/T34A-C4, stably transfected with an inducible dominant-negative survivin gene under the control of a tetracycline-regulated (tet-off) promoter. PDT treatment of melanoma cells expressing the dominant-negative survivin resulted in increased cleavage of the caspase substrate poly(ADP-ribose) polymerase, apoptosis, and cytotoxicity when compared with results following PDT of the same melanoma cell line expressing wild-type survivin. These results show for the first time that targeting survivin and possibly other Hsp-90 client proteins improves in vitro PDT responsiveness and suggest that manipulation of the antiapoptotic pathway maintained by survivin may enhance PDT-mediated cancer therapy.

Introduction

Photodynamic therapy (PDT) is used to treat a variety of solid tumors, including malignancies of the lung, esophagus, brain, prostate, head and neck, and skin (1–3). The Food and Drug Administration–approved photosensitizer Photofrin as well as several second-generation photosensitizers are used in a variety of clinical trials. PDT induces the photochemical generation of cytotoxic reactive oxygen species within the irradiated tissue, which leads to tumor cell death through necrosis, apoptosis, and/or autophagy (4–7). Vascular injury, tissue hypoxia, and inflammatory reactions with concomitant expression of growth factors, matrix metalloproteinases, cytokines, and prostaglandins are observed within the treated tumor microenvironment (8–12). These treatment-related reactions can be associated with an angiogenic and/or survival phenotype that may play a role in tumor recurrences following PDT and highlight the need to more fully understand the molecular responses initiated by PDT.

We recently observed that PDT induces increased expression and phosphorylation of survivin in murine cancer cells and tumors. Survivin is a member of the inhibitor of apoptosis (IAP) family and has been shown to suppress apoptosis and regulate cell division (13–15). Phosphorylation of survivin extends the lifetime of this IAP protein and allows for enhanced protein activity (16–18). Survivin is detected in malignant lesions and in fetal tissue but is absent in most adult differentiated tissues (17). A variety of pharmacologic and environmental stimuli can increase survivin expression, including UVB exposure, chemotherapeutic agents, hypoxia, and vascular injury (17). Levels of survivin in clinical tumor samples correlate inversely with patient prognosis, and up-regulation of survivin is associated with resistance to chemotherapy and radiotherapy (15, 19). The cytoprotective role of survivin also extends beyond the tumor cell population to involve endothelial cells within the tumor microenvironment (17, 20).

Survivin binds to the 90-kDa heat shock protein (Hsp-90) in cells and is therefore considered a Hsp-90 client protein (21). Hsp-90 provides the necessary intracellular chaperone environment for proper folding and maturation of a variety of client proteins, many of which are involved in signal transduction and cell proliferation (17). Geldanamycin and its clinically related derivative, 17-allylamino-17-demethoxygeldanamycin (17-AAG), bind to the ATPase binding region of Hsp-90 and inhibit client protein binding (22). Disrupting the chaperone function of Hsp-90 results in destabilization and degradation of its client proteins and, in the case of survivin, can initiate apoptosis and suppress cell proliferation (21, 23, 24).

The goal of the current study was to determine if disruption of the cellular function of survivin would modify the apoptotic and cytotoxic responses of PDT. We first examined whether
photosensitizers with differing subcellular localization properties induced PDT-mediated expression of survivin and its phosphorylated form in treated cells. We next used pharmacologic and genetic approaches to determine if attenuating survivin levels and activity would modify the effectiveness of PDT in human cancer cells. Our results indicate that both porphyrin-based (Photofrin) and chlorin-based (NPe6) photosensitizers induced increased expression and phosphorylation of survivin. Our results also show that inhibition of survivin increased PDT-mediated apoptosis and cytotoxicity. These findings suggest that a combined modality approach involving PDT and survivin inhibition may enhance PDT effectiveness.

Materials and Methods

**Drugs.** Photofrin porfimer sodium (a gift from Axcan Scandipharma, Inc.) was dissolved in 5% dextrose in water to make a 2.5 mg/mL stock solution and then stored at −20°C. Mono-l-aspartyl chlorin e6 or NPe6 (a gift from Porphyrin Products, Inc.) was dissolved in saline to make a 2.5 mg/mL stock solution and then stored at −20°C. Stock solutions of photosensitizers were diluted in culture medium before use. 17-AAG (purchased from A.G. Scientific, Inc.) was dissolved in DMSO at a concentration of 1 mmol/L and stored at −20°C. The stock solution of 17-AAG was diluted in culture medium immediately before use.

**Cell culture and tumor models.** Mouse breast cancer (BA) and human breast cancer (BT-474) cells were grown as monolayer cultures in RPMI 1640 or DMEM, respectively, supplemented with 10% FCS and antibiotics. BA tumors were generated by s.c. trochar injection of 1 mm³ pieces of tumor to the hind right flank of 8- to 12-week-old female C3H/HeJ mice (25). The human melanoma cell line (YUSAC2/T34A-C4) stably transfected with an inducible phosphorylation-defective, dominant-negative survivin (Thr34 > Ala) mutant controlled by the tetracycline (tet) promoter was kindly provided by D. Grossman (Huntsman Cancer Institute, Salt Lake City, UT) and maintained in DMEM containing 5% FCS, 0.5 μg/mL tetracycline (Sigma), 1.5 mg/mL G418 (Omega Scientific, Inc.), 2 mmol/L sodium hydroxide, glutamine, and antibiotics (18).

**In vitro and in vivo treatment protocols.** For *in vitro* treatments, cells were seeded in Petri dishes and incubated either for 24 h in complete growth medium (BA and BT-474 cells) or for 48 h in complete selection medium containing G418 in the presence or absence of tet (YUSAC2/T34A-C4 cells) to allow for attachment. Photosensitization experiments were done as reported previously (10, 12). Briefly, cells were incubated in the dark in medium containing 5% serum together with either Photofrin or NPe6 (25 μmol/L) for 16 h at 37°C. This medium was then removed and the cells were then incubated for 30 min in fresh growth medium containing 10% serum (BT cells) or 5% serum (YUSAC2 cells) and then rinsed in medium without serum before light treatment. Photofrin-incubated cells were exposed to broad-spectrum red light (570–650 nm) generated by a parallel laser (Miravant Medical Technologies) using a dose rate of 20 mW/cm². Treated cells were refed with complete growth medium in the presence or absence of either 17-AAG (BT-474 cells) or tet (YUSAC2/T34A-C4 cells). Survival of BT-474 and YUSAC2/T34A-C4 cells was measured 24 h after photosensitization using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazo- lium bromide (MTT) assay. Control and treated cells were transferred into flat-bottomed 96-microwell plates (5 × 10⁴ per well), and MTT reagent was added to a final concentration of 5 μmol/L (26). The cells were incubated at 37°C for 4 h and cleavage of MTT was determined by absorbance at 570 nm using a microplate reader. Survival of treated cells was normalized to control cells. Eight samples were analyzed for each dose per experiment, and all experiments were done in duplicate. *In vivo* PDT treatment of BA tumors involved an i.v. injection of Photofrin (5 mg/kg) followed 24 h later with tumor irradiation using a 630 nm diode laser (Diomed). Light was delivered via a quartz fiber microlens system. A light dose rate of 75 mW/cm² and a total light dose of 50 J/cm² were used for *in vivo* PDT treatments.

**Detection of apoptosis.** Cellular apoptosis was evaluated using a Cell Death Detection ELISA Plus kit (Boehringer Mannheim) as reported previously. This assay quantifies mononucleosomes and oligonucleosomes from cell lysates using monoclonal antibodies targeting DNA and histones in a quantitative photometric sandwich enzyme immunoassay (27). Cells were analyzed 24 h after treatment. Two samples were analyzed for each dose and time point per experiment, and all experiments were done in duplicate. Apoptotic enrichment factors were calculated from absorbance ratios of treated versus control cells. Results were normalized for protein concentrations.

**Western immunoblot analysis.** Protein expression was documented by Western immunoblot analysis (10, 12). Cells were collected 24 h after PDT and sonicated in 1× cell lysis buffer (Cell Signaling Technology, Inc.) containing phenylmethylsulfonyl fluoride. BA tumors were homogenized with a polytron in 1×reporter lysis buffer (Promega). Protein samples were size separated on discontinuous polyacrylamide gels (7.5–14%) and transferred overnight to nitrocellulose membranes. Filters were blocked with 5% nonfat milk and then incubated overnight with either mouse monoclonal anti-poly(ADP-ribose) polymerase (PARP; clone C2; Novus Biologicals), mouse monoclonal anti-cleaved PARP (Asp²²⁵; Cell Signaling Technology), rabbit polyclonal antibody to phosphorylated survivin (Thr²³⁷; Novus Biologicals), rabbit polyclonal antibody to survivin (Chemicon International), rabbit polyclonal antibody to phosphorylated Akt (Ser⁴⁷³; Cell Signaling Technology), rabbit polyclonal antibody to Sel-Hsp-90 (SPA-846; Stressgen Bioreagents Corp.), or mouse monoclonal anti-Bcl-2 (clone C-2; Santa Cruz Biotechnology). Filters were then incubated with either an anti-mouse or an anti-rabbit peroxidase conjugate (Sigma), and the resulting complexes were visualized by enhanced chemiluminescence autoradiography (Amersham Life Science). Protein loading was evaluated by subsequently incubating the filters with a mouse monoclonal anti-actin antibody (clone C-4; ICN).

**Survivin protein measurements.** Quantitative levels of survivin were obtained for control and treated BT-474 cells using the human Total Survivin TiterZyme Enzyme Immunoassay (EIA) kit (Assay Designs). Protein concentrations were determined from cell lysates, and survivin content was calculated per mg of total protein.

**Photofrin uptake.** Photofrin concentrations were measured in YUSAC2/T34A-C4 cells growing in the presence or absence of tet using absorption spectroscopy. Photofrin was extracted from cells by sonication in 0.2 N NaOH. Porphyrin concentrations were determined from calibration curves using absorption ratios at 390/470 nm (28). Results were expressed as μg Photofrin per mg of total protein.

**Statistics.** Statistical significance of EIA, ELISA, and MTT results was determined using a two-tailed Student’s *t* test. Results with *P* < 0.05 were considered significant.

**Results**

**PDT induces expression of survivin and phosphorylated survivin in treated cancer cells and tumors.** A variety of anticancer agents and exogenous stress conditions, including exposure to Adriamycin, Taxol, and UVB, can increase survivin expression (14–18). We initially examined whether PDT influenced the expression profiles of survivin and phosphorylated survivin in mouse BA mammary carcinoma cells and tumors. Figure 1A shows that exposure of BA cells to an IC₅₀ dose of Photofrin-mediated PDT induced an increase in both survivin and phosphorylated survivin expression when analyzed 24 h after treatment. Similar results were observed when BA tumors growing in C3H mice were treated with Photofrin-mediated PDT and assayed for survivin levels 24 h after treatment as shown in *Fig. 1B.*

**17-AAG attenuates the expression of Hsp-90 client proteins, including survivin, and increases apoptosis and cytotoxicity in PDT-treated cells.** Survivin is a Hsp-90 client protein and binding of survivin to Hsp-90 allows for proper protein folding and
maturation (21, 23, 24). The geldanamycin derivative 17-AAG leads to proteasome degradation of Hsp-90 client proteins by competing for the binding of these proteins to Hsp-90. We examined cellular responses following treatment protocols involving PDT with or without 17-AAG using human BT-474 breast cancer cells. Expression of apoptotic-related proteins, including some Hsp-90 client proteins, is shown in Fig. 2A and B for BT-474 cells treated with either Photofrin- or NPe6-mediated PDT. At the time of collection, we observed increased expression of survivin and phosphorylated survivin. PDT did not alter the levels of phosphorylated Akt above control photosensitizer alone levels. Combining PDT with 17-AAG increased the cleavage of PARP from the native 116-kDa enzyme to an 89-kDa fragment, enhanced degradation of the Bcl-2 protein, and decreased expression of the native 116-kDa enzyme to an 89-kDa fragment, enhanced degradation of the Bcl-2 protein, and decreased expression of survivin, phosphorylated survivin, and phosphorylated Akt. Quantitative levels of survivin in PDT-treated BT-474 cells were analyzed using an ELISA assay, and Fig. 2C and D showed a 3-fold increase when compared with control levels. Cellular incubation with 17-AAG resulted in decreased survivin levels, which were not statistically significant, but this trend agreed with the results obtained by Western immunoblot analysis. Cellular exposure to 17-AAG did not modulate the expression of Hsp-90 or actin.

Apoptosis and cytotoxicity were determined for control and treated cells 24 h after Photofrin- or NPe6-mediated PDT as shown in Fig. 3. Both PDT alone and treatment with 17-AAG alone caused measurable levels of apoptosis (Fig. 3A and B) and cytotoxicity (Fig. 3C and D). Combining PDT with 17-AAG further increased apoptosis and cytotoxicity. Incubation of cells with DMSO at concentrations used to dissolve 17-AAG did not affect molecular or cellular responses to PDT treatments (data not shown).

Inducible expression of phosphorylation-defective, dominant-negative survivin in YUSAC2/T34A-C4 cells increases apoptosis and cytotoxicity following PDT. YUSAC2/T34A-C4 melanoma cells expressing an inducible (tet-off) survivin mutant Thr34→Ala were used to confirm the involvement of the survivin pathway in modulating the apoptotic response to PDT (18). Cells were grown either in the presence of tet, which allows expression of biologically active Thr34-phosphorylated survivin, or in the absence of tet, which induced the expression of the survivin mutant that cannot be phosphorylated at Thr34 due to the conversion of threonine to alanine. Figure 4A (top) shows the expression pattern for survivin and phosphorylated survivin in this experimental model. In the absence of tet, the YUSAC2/T34A-C4 cells express a 16.5-kDa mutant survivin, whereas minimal survivin expression was observed in the presence of tet. In agreement with previous reports, there was a significant reduction in the expression of Thr34-phosphorylated survivin for cells cultured in the absence of tet compared with cells cultured in the presence of tet. Growing the YUSAC2/T34A-C4 cells in the presence or absence of tet did not alter the uptake of Photofrin as shown in Fig. 4A (bottom) Apoptosis measurements were obtained 24 h after Photofrin-mediated PDT for YUSAC2/T34A-C4 cells cultured either in the presence or in the absence of tet. Figure 4B (top) shows that apoptotic indexes followed a dose-dependent pattern, and levels were significantly higher under “tet-off” culture conditions when the dominant-negative survivin was expressed. These results agree with Western blot analysis data shown in Fig. 4B (bottom) where a dose-dependent cleavage of PARP was observed following PDT only in the absence of tet, conditions that inhibit survivin phosphorylation. PDT treatment in the presence of tet (leading to survivin phosphorylation) resulted in reduced apoptosis and cleavage of PARP. Cytotoxicity measurements for PDT-treated YUSAC2/T34A-C4 cells agreed with observed apoptosis levels. Figure 4C shows that there was an increase in cytotoxicity for cells cultured in the absence of tet when the dominant-negative survivin mutant was overexpressed.

Discussion

We show for the first time that PDT increases the expression of survivin and phosphorylated survivin in malignant cells and tumors. Overexpression of survivin is associated with the inhibition of apoptotic pathways, playing a primary role in antagonizing mitochondrial-dependent apoptosis (14, 15). In addition to modulating apoptosis, growing evidence indicates that survivin also is involved in regulating cell division. A critical requirement for survivin stabilization and function is phosphorylation on Thr34 by the mitotic kinase p34<sup>Bud2</sup>-cyclin B1 (16, 29). Signaling molecules up-regulated by PDT, including phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase, hypoxia-inducible factor-1α, activator protein-1, and nuclear factor-κB, are inducers of the expression of survivin (8, 9). Likewise, inflammatory cytokines, vascular endothelial growth factor, vascular injury, and hypoxia are associated with increased expression
and/or stability of survivin and these responses are also increased following PDT (10–12). Studies are currently under way to identify exactly how PDT induces expression and activation of survivin.

Survivin is undetectable in most terminally differentiated normal tissues but is highly expressed in many malignancies (30). Patients with tumors expressing high survivin levels generally have a poor prognosis concomitant with increased rates of recurrence, resistance to radiotherapy and chemotherapy, and a reduced apoptotic index (19, 20, 30). Therapeutic procedures targeting survivin could therefore reduce the survival phenotype observed in tumor tissue without affecting normal tissue (17). In this regard, a variety of strategies are being tested to target survivin, including (a) generating antigen-specific cytotoxic T cells against survivin peptides; (b) use of antisense molecules, ribozymes, and dominant-negative mutants of survivin; (c) pharmacologic inhibition of survivin phosphorylation; and (d) pharmacologic inhibition of survivin binding to Hsp-90 (21, 23, 30).

Our discovery that survivin expression and phosphorylation are increased following PDT led to the hypothesis that survivin modulates PDT responsiveness.

To address this issue, we used both a pharmacologic and a genetic approach to examine the effects of modulating survivin expression and phosphorylation on PDT responsiveness. We also examined photosensitizers with differing subcellular localization properties and observed that PDT using either Photofrin or NPe6 induced expression and phosphorylation of survivin (6). Survivin is a Hsp-90 client protein and Hsp-90 promotes the proper folding, maturation, and transport of client proteins within a cell (21). A clinically relevant derivative of the benzoquinone ansamycin antibiotic geldanamycin, 17-AAG, binds to the ATP/ADP pocket of Hsp-90, inhibits Hsp-90 function, and leads to the disassociation and degradation of client proteins (31). We observed that 17-AAG alone induced measurable cytotoxicity in human BT-474 breast cancer cells, and this observation agrees with previous reports for various human cancer cell lines exposed to this geldanamycin derivative (31, 32). Exposure of PDT-treated cells to 17-AAG decreased levels of survivin, Akt, and
Bcl-2. This procedure also led to increased PARP cleavage, apoptosis, and cytotoxicity. Interestingly, this response was observed using photosensitizers that primarily localize either to the mitochondria (Photofrin) or to the lysosomes (NPe6). Our observations agree with studies showing 17-AAG potentiating the radiation response of tumor cells via an apoptotic mechanism as well as reducing IC_{50} levels of chemotherapeutic agents in tumor cells (31, 32).

Our experiments combining PDT with 17-AAG clearly show that disrupting Hsp-90 client protein interaction can decrease survivin levels and increase both apoptosis and cytotoxicity. 17-AAG is in clinical trials for the treatment of a variety of tumors, and therefore, combining PDT with 17-AAG may be clinically relevant (30, 33). However, there are several Hsp-90 client proteins in addition to survivin that could be involved in modulating PDT, and therefore, this pharmacologic approach is a nonspecific targeting protocol. To confirm a specific role for survivin in modulating PDT, we used a genetic approach involving expression of an inducible dominant-negative survivin to determine if selective inhibition of this IAP protein could modulate PDT responsiveness. Previous studies have shown that suppression of survivin phosphorylation by the inducible expression of the phosphorylation-defective, dominant-negative survivin gene enhances tumor cell apoptosis and inhibits tumor growth (18). Our results show that selective targeting of the survivin pathway can increase PDT-mediated apoptosis and cytotoxicity. Studies are in progress to determine if PDT enhances survivin phosphorylation in normal cells and tissue.

In summary, although PDT continues to be an effective clinical option for treating solid tumors, it also induces considerable stress within the tumor microenvironment. This includes both oxidative stress produced by the photochemical generation of reactive oxygen species and hypoxia resulting from the rapid vascular damage produced by PDT and/or by the photochemical consumption of oxygen (8). A consequence of PDT-mediated stress is the induction of a survival phenotype associated with increased expression of angiogenic growth factors, cytokines, proteinases, and antiapoptotic molecules. Our increasing knowledge of PDT responses at a molecular level provides significant opportunities to further improve the

**Figure 3.** 17-AAG increases apoptosis and cytotoxicity in PDT-treated human BT-474 breast cancer cells. Apoptosis levels were measured 24 h after Photofrin-PDT (A) or NPe6-PDT (B) either in the absence (–) or in the presence (+) of 1 μmol/L 17-AAG. Apoptotic indexes were determined using the Cell Death Apoptosis Detection ELISA Plus kit. Columns, mean (n = 2); bars, SE. *, P < 0.05 (PDT + 17-AAG versus PDT). Cytotoxicity in BT-474 cells was measured using a MTT cell proliferation assay 24 h after Photofrin-PDT (C) or NPe6-PDT (D) either in the absence (–) or in the presence (+) of 1 μmol/L 17-AAG. Columns, mean (n = 2); bars, SE. *, P < 0.05 (PDT + 17-AAG versus PDT).
therapeutic effectiveness of PDT. Clinically relevant inhibitors that block angiogenesis, prostanoid release, and proteinase activation can enhance PDT responsiveness in the laboratory because there is a differential effect on tumor tissue compared with normal tissue (8, 12). The data obtained in the current study suggest that strategies to decrease the survival phenotype associated with PDT-mediated survivin expression may also improve PDT effectiveness.

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