Photodynamic therapy of human breast cancer xenografts lacking caspase-3

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Abstract

The human breast cancer cell line MCF-7 is deficient in procaspase-3 and in caspase-3-dependent steps in apoptosis due to deletion of the CASP-3 gene. We previously found that the cells transfected with empty vector (MCF-7v cells) were considerably less sensitive to photodynamic treatment in vitro with the phthalocyanine photosensitizer Pc 4 than were the cells stably transfected with human procaspase-3 cDNA (MCF-7c3 cells); however, overall cell killing, as determined by a clonogenic assay, was not affected by the presence of procaspase-3. The present study was undertaken to determine whether photodynamic therapy (PDT) in vivo was dependent on the ability of the cells to carry out the late steps in apoptosis that are catalyzed by this caspase. Xenografts of MCF-7 cells and the isogenic-derived MCF-7v and MCF-7c3 cells were generated in female athymic nude mice implanted with an estrogen pellet. MCF-7c3 xenografts, but not those of the other two lines, continued to express procaspase-3, as revealed by Western blots of proteins from the cells and the xenografts. When the xenografts reached 50–120 mm\textsuperscript{3}, some were treated with PDT (1 mg/kg Pc 4 i.v. followed 48 h later by 150 J/cm\textsuperscript{2} light at 672 nm and 150 mW/cm\textsuperscript{2}), while others served as controls (no treatment, light alone, or Pc 4 alone). All Pc 4-PDT-treated tumors and none of the controls exhibited either complete or strong partial responses, and complete responses were durable for the entire observation period of 16 days. The responses were not dependent upon the presence of procaspase-3 in the xenografts. The results indicate that the rapid response of Pc 4-PDT-treated tumors in vivo is not due to their ability to carry out the major caspase-3-mediated late steps in apoptosis. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

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1. Introduction

Photodynamic therapy (PDT) is a promising cancer treatment in which a photosensitizing drug accumulates in tumors and is subsequently activated by visible light of an appropriate wavelength matched to the absorption spectrum of the photosensitizer [1,2]. The energy of the activated photosensitizer is subsequently transferred to molecular oxygen to produce singlet oxygen and other reactive oxygen species that produce cell death and tumor ablation. In response to PDT, many cells in culture or within experimental tumors are eliminated by apoptosis, a programmed mechanism of cell death in which cells
undergo characteristic biochemical and morphological changes [3,4]. Mitochondria are central clearinghouses for signaling apoptosis [5]. PDT with photosensitizers that localize in mitochondria, such as the phthalocyanine Pc 4, bypass many of the premitochondrial steps of apoptosis initiated by other agents, such as ionizing radiation or tumor necrosis factor (TNF-α), directly targeting mitochondria.

Photodamage from Pc 4-PDT causes prompt release of cytochrome c from the mitochondria into the cytoplasm and activation of caspases-9 and -3, proteases frequently responsible for cell degradation during in vitro apoptosis [6–10]. The importance of this pathway has recently been demonstrated in in vitro studies with the human breast cancer cell line MCF-7 that lacks procaspase-3 and is relatively resistant to the induction of apoptosis by Pc 4-PDT [11] as well as by several other apoptosis-inducing agents [12–14]. Stable transfection of procaspase-3 resulted in a cell line (MCF-7c3) that readily undergoes apoptosis in response to Pc 4-PDT and is more sensitive to cell killing by Pc 4-PDT, when judged by the WST-1 tetrazolium dye reduction assay, than the companion cell line (MCF-7v) transfected with an empty vector. However, when cell death was measured by a clonogenic assay, the two cell lines were equally sensitive to Pc 4-PDT and differ as a function of the presence of caspase-3.

Apoptosis has been found in all tumor systems where it has been sought after PDT (reviewed in Refs. [3,15]), suggesting that the ability of PDT to rapidly generate apoptotic cells in tumors may in part explain the rapid ablation of tumors that occurs after PDT. The purpose of the present study was to determine if the caspase-3-dependent late steps of apoptosis are important for the response of human breast tumor cells to Pc 4-PDT in vivo. For this purpose, MCF-7 cells were grown as xenografts in athymic nude mice and their PDT response monitored.

2. Materials and methods

2.1. Cell culture

MCF-7v, MCF-7c3, and untransfected MCF-7 cells were cultured in RPMI 1640 medium supplemented with glutamine and 10% fetal bovine serum. MCF-7v and MCF-7c3 were grown in the presence of 2 μg/ml puromycin, as previously described [11].

2.2. Preparation of xenografts

For the generation of xenografts, 4-week-old female athymic mice (athymic nu/nu; Ireland Comprehensive Cancer Center Athymic Animal Facility), about 20–22 g body weight, were used. Animals were implanted with estrogen pellets (60-day-release, 17β-estradiol, 1.7 mg/pellet; Innovative Research of America, Sarasota, FL, USA), and immediately thereafter 10^7 cells in 0.2 ml serum-free MEM were injected s.c. into both the anterior forelimb and one mammary fat pad in each mouse. Animals were maintained under pathogen-free conditions. Animals were treated when xenografts reached about 50–120 mm^3, as described below.

2.3. PDT

Pc 4 was formulated as previously described [16]. For PDT, three animals of each group of five were given 1 mg/kg of Pc 4 by tail vein injection, and 48 h later (defined as Day 1), a 1-cm area encompassing the five dorsal tumors in each group received 150 J/cm^2 of 672-nm light at 150 mW/cm^2. The tumors in the mammary fat pads served as dark controls. Growth of the xenografts was monitored by measuring tumor size in three dimensions using a caliper. Tumor volume (V) was determined by the following equation: \( V = (L \times W \times H) \times 0.5236 \), where \( L \) is the length, \( W \) is the width, and \( H \) is the height of the xenograft [17].

2.4. Statistical analysis of tumor response

Tumor volume, measured as a percentage of initial (day 1) volume on days 3, 5, 8, 10, 15, and 17, was obtained for four treatment groups that were designated Control, Light, Pc 4, and Pc 4-PDT. An analysis of repeated measures for mixed data was implemented using SAS PROC MIXED [18] with treatment group being a fixed effect and time as the repeated measure. The analysis was stratified by cell lines (MCF-7, MCF-7v, and MCF-7c3). Three contrasts between mean levels of Pc 4-PDT and each of Control, Light, and Pc 4 treatments were estimated. Initial
modeling included an interaction term between time and treatment, which was found to be not statistically significant and was dropped from the model.

2.5. Western blot analysis

Animals were sacrificed 60 days after implantation of estrogen pellets, and the xenografts were quickly resected and frozen in liquid nitrogen. Xenografts were pulverized using a mortar and pestle on dry ice. Cell pellets and pulverized xenografts were lysed by sonication in a solution comprising 0.5% sodium deoxycholate, 0.2% sodium dodecyl sulfate (SDS), 1% Triton X-100, 1% Nonidet-P40, 5 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM phenylmethylsulfonyl fluoride in ice-cold phosphate buffered saline (PBS) (all reagents from Sigma Chemical Co., St. Louis, MO, USA). Samples (50 μg protein determined by Dc BioRad protein assay from BioRad, Hercules, CA, USA) were separated by polyacrylamide gel electrophoresis consisting of a 5% (w/v) acrylamide stacking gel and a 12.5% (w/v) acrylamide separating gel containing 0.1% SDS. The running buffer contained 0.1% SDS, 25 mM Tris, and 250 mM glycine, pH 8.3. Electrophoretic fractionation was carried out at a constant current of 15 mA until bromophenol blue migrated about 10 cm. Proteins were electrotransferred onto an Immobilon p15 membrane (Millipore Corp., Bedford, MA, USA). The filters were blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS, then incubated overnight at 4°C with 1 μg/ml primary antibody directed to human procaspase-3 from Transduction Laboratories (Lexington, KY, USA); this antibody did not cross-react with mouse procaspase-3. Actin was used as internal standard for protein loading. The secondary antibody was horseradish peroxidase-conjugated anti-mouse immunoglobulin (1:1000 in blocking solution). Bands were visualized with enhanced chemiluminescence reagent and subsequent exposure to hyperfilm-enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA).

3. Results

3.1. Growth of the xenografts

Growth of the three MCF-7 cell lines as xenografts was very slow, with those derived from the parental MCF-7 cell line reaching 50–120 mm^3 about 22 days after injection and those derived from the MCF-7v and MCF-7c3 cell lines requiring 40–45 days after injection to reach the same size range. It would not be expected that the presence or absence of procaspase-3 would affect xenograft growth rate, and that was borne out by the observation that MCF-7v and MCF-7c3 xenografts grew at the same rate. The slower in vivo growth of the transfected cells may be related to biochemical processes resulting from transfection per se or to the use of puromycin during in vitro culture of the cells.

3.2. Procaspase-3 expression in the xenografts

In order to evaluate the role of caspase-3 in the response of the xenografts to Pc 4-PDT, it was necessary to demonstrate that the xenografts prepared from MCF-7c3 cells retained the ability to express procaspase-3 after growth in athymic nude mice. This was a concern, because the transfected cells were cultured in vitro in the presence of puromycin to continuously select for cells that retained the transfected DNA, but no puromycin was given to the mouse hosts. Without the selection pressure, it was possible that non-expressing cells would eventually dominate the xenografts. Therefore, proteins were solubilized from cell cultures and from xenografts prepared from each of the three lines and subjected to Western blot analysis. Fig. 1A demonstrates that for both cells and xenografts, only protein samples from MCF-7c3 cells exhibited a band that could be detected by the anti-human procaspase-3 antibody, although all three lines were positive for the presence of actin. We have previously observed [17] two major bands at Mr 66,000–75,000 and 50,000 and other minor bands at Mr 55,000, 49,000, and 25,000 resulting from reactivity of the secondary antibody with IgG in the SW480 colon cancer xenograft samples. Therefore, we have evaluated reactivity with the secondary antibody alone in the MCF-7 xenografts to assess the extent of overlap with the procaspase-3 band. Although reaction with IgG heavy and light chains was detected in variable amounts in each animal (Fig. 1), these were distinct from the bands for procaspase-3 and actin. Therefore, it is clear that human procaspase-3 is expressed in the
xenografts derived from MCF-7c3 cells, and not in the other xenografts.

3.3. Response of the xenografts to Pc 4-PDT

Tumors were treated approximately 22 (MCF-7) or 44 (MCF-7v and MCF-7c3) days after implantation of the estrogen pellets and injection of the cells, when they reached approximately 50–120 mm³. Because the xenografts prepared from the untransfected MCF-7 cells grew slightly faster than those from the other two lines, they were treated earlier than the other two sets of xenografts. For treatment, the animals received Pc 4 (1 mg/kg) then light 48 h later (Day 1), and tumor size was measured over the next 16 days. For each tumor, the data have been normalized to the size at the time of laser irradiation (i.e. 100%). The results of the treatments are shown in Fig. 2. Tumors that received either no treatment, Pc 4 alone or light alone maintained their size or grew slightly during the period of observation. In contrast, either a complete or partial response was obtained in all of the Pc 4-PDT-treated tumors, regardless of the cell source. When the data for the treated tumors were expressed as % of the tumor size at the time of PDT, an average of 80, 95, and 70% reduction in tumor size was obtained in MCF-7, MCF-7v, and MCF-7c3 xenografts, respectively, by 3 days post-PDT, and complete responses were durable during the observation period. Pellets providing a 60-day estrogen supply determined the length of the experiment. We elected not to implant a second estrogen pellet in these animals, because our primary concern was the early response of the xenografts to Pc 4-PDT.

There was no statistically significant interaction between time and treatment for each of the cell lines and this term was dropped from the model. Estimates of the contrast between Pc 4-PDT and each of the other three treatments indicated a statistically significant ($P < 0.0001$) difference between the mean level of tumor volume, measured as a percentage of initial volume immediately prior to treatment, for Pc 4-PDT and each of the Control, Light, and Pc 4 treatments.

4. Discussion

The generation of xenografts in athymic nude mice from estrogen-dependent human breast tumor cells requires that an estrogen pellet be implanted in the animals. Even with the augmented estrogen, it is well established that growth of breast cancer xenografts is very slow [19,20]. Therefore, the animals were treated when the xenografts had reached 50–120 mm³. In spite of the long time from implantation until study, the MCF-7c3 xenografts, like the cells...
from which they were derived, continued to express the human procaspase-3 transgene, and no evidence for procaspase-3 was found in either of the other two cell lines or the xenografts made from them (Fig. 1).

The tumor responses were consistent for each treatment and across xenograft types (Fig. 2). The six repeated measurements for each animal generated considerably more data than would have been realized from a simple measure from each animal. An analysis of repeated measures for the treatment groups, considered as a fixed effect, and for times after treatment, considered as the repeated measure, allows an analysis of the data in their entirety taking into account the existing correlations between measurements taken at different times for each animal. Such an analysis, then, has a larger number of degrees of freedom for the error estimates leading to improved power. Specifically, there were 60 observations available for each xenograft type (six time points, ten tumors), which afforded sufficient power to statistically discern the large observed differences. Moreover, it may be noted from Fig. 2 that the variation within each treatment group is considerably smaller than the variation between the treatment groups. This contributes further to statistical power.

The tumors that received only Pc 4 or only light increased in volume at a similar rate as untreated tumors during the observation period (Fig. 2). In contrast, all of the tumors that received both Pc 4 and light exhibited a dramatic decrease in size within the first 1–3 days after irradiation. Therefore, the initial response of MCF-7 human breast tumor xenografts to Pc 4-PDT was not dependent on the ability of the tumor cells to carry out the normal terminal stage of apoptosis, in which the major executioner caspase, caspase-3, cleaves key structural proteins and enzymes and activates endonucleolytic DNA scission and chromatin condensation. The data of Fig. 2 are consistent with our in vitro data demonstrating that the lethal damage inflicted by Pc 4-PDT was upstream and independent of the activity of caspase-3 [11]. If apoptosis is important for the response of tumors to Pc 4-PDT, we suggest that early steps in the process are more relevant than the late ones.

The response of tumors to PDT is a result of a combination of direct damage to the malignant cells, damage to the tumor vasculature that indirectly kills

Fig. 2. Response of xenografts derived from MCF-7, MCF-7v, and MCF-7c3 cells to Pc 4-PDT. Each panel represents data from five mice, each implanted with two xenografts. Three animals of each group were given Pc 4 by injection, and 48 h later all of the dorsal tumors were irradiated. The five tumors of each set in the mammary fat pads served as dark controls. Tumor size was followed over the next 16 days and is recorded as % of the size at the time of photolysis (Day 1). In each panel, the symbols and error bars represent the average and standard deviation, respectively, of the normalized sizes of two control tumors (mammary fat pad, no Pc 4, no light, ○); three tumors from animals given Pc 4 alone (mammary fat pad, no light, ▼); two tumors exposed to light alone (dorsal, no Pc 4, ◊), and three PDT-exposed tumors (dorsal, Pc 4, light, ▽). Complete responses (no measurable tumor) were obtained in two of the three PDT-treated MCF-7v xenografts.
the malignant cells, and inflammatory/immune responses triggered by the release of various cytokines from the treated tissue and the recruitment of neutrophils, macrophages, and other inflammatory cells to the tumor [1,21–23]. The contribution of each of these mechanisms to the overall tumor response varies with the photosensitizer. For Pc 4-PDT, extensive direct malignant cell killing has been noted (C. Anderson and C. Elmets, unpublished). However, one explanation for the efficient tumor ablation that is independent of caspase-3 activity in the MCF-7 xenografts may be that the controlling factors are late-arising vascular or inflammatory responses. Therefore, the dominance of factors external to the MCF-7 cells in the observed responses cannot be ruled out. However, immune responses would be expected to be sub-optimal in the athymic nude mice.

The study of the response of MCF-7v and MCF-7c3 cells to Pc 4-PDT in vitro indicated that the death of the cells was due to lethal photodamage that was independent of caspase-3. There are several possible candidates that could be regarded as the critical lethal upstream step. First, PDT with Pc 4 and certain other photosensitizers cause immediate photodamage to the anti-apoptotic protein Bcl-2 [24,25]. Bcl-2 photodamage was found in all cells examined, including MCF-7v and MCF-7c3 cells [25]. Once Bcl-2 is damaged, it may no longer be able to protect the mitochondrial membrane, resulting in the loss of mitochondrial membrane potential and release of cytochrome c and other pro-apoptotic proteins into the cytosol. Thus, it is possible that sufficient photochemical damage to Bcl-2 can guarantee that a cell will die. Second, the release of cytochrome c into the cytosol can itself be a lethal event [26], either because it initiates the caspase-9- and 3-dependent pathway for apoptosis or because it results in mitochondria that are deficient in respiratory capacity and in the ability to generate adenosine triphosphate (ATP) [6,26]. Thus, we suggest that Pc 4-PDT-treated cells are doomed as a result of the initial mitochondrial damage, and this may also be the case for cells within xenografts treated with Pc 4-PDT. Within a tumor, the Pc 4-PDT-damaged cell may signal its doomed status to the plasma membrane in a manner that ensures its efficient engulfment by recruited phagocytic cells, whether or not it continues to undergo degradation by the normal apoptotic pathway. The invading host cells that phagocytize Pc 4-PDT-damaged cancer cells could potentially provide the caspase-3 and other late apoptotic enzymes needed to complete the digestion of the cancer cells.

The results of this study suggest that caspase-3 does not sensitize MCF-7-derived xenografts to Pc 4-PDT in terms of the kinetics of the reduction of tumor mass or the durability of the response. It will be of interest in future work to study the response of tumor models deficient in other steps in apoptosis to further refine the identification of the critical lethal damage.

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References


