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Advances in Brief

Up-Regulation of Clusterin during Phthalocyanine 4 Photodynamic Therapy-mediated Apoptosis of Tumor Cells and Ablation of Mouse Skin Tumors[1]

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Abstract

Photodynamic therapy (PDT) using the silicon phthalocyanine photosensitizer Pc 4 is an oxidative stress associated with the induction of apoptosis in many cancer cells *in vitro* and *in vivo*. The mechanisms of PDT-induced tumor cell killing leading to apoptosis are incompletely understood. Clusterin, a widely expressed glycoprotein, is induced in tissues regressing as a consequence of oxidative stress-mediated cell death. Treatment of apoptosis-sensitive human epidermoid carcinoma cells (A431) with PDT resulted in significant up-regulation of clusterin with a maximum at 12 h after treatment, whereas clusterin levels in Pc 4-PDT-treated, apoptosis-resistant, radiation-induced fibrosarcoma (RIF-1) cells remained unchanged. The i.v. administration of Pc 4 to mice bearing chemically or UVB radiation-induced skin papillomas, followed by light application, led to increased clusterin protein expression, peaking 24 h after the treatment, when tumor regression was apparently visible. These data, for the first time, demonstrate the involvement of clusterin in PDT-mediated cell death and during tumor regression. This may have relevance in improving the efficacy of PDT using pharmacological inducers of clusterin.

Introduction

PDT[3] represents a relatively novel therapeutic procedure that has emerged as an effective modality to treat a variety of solid and superficial tumors. Basic and clinical PDT research culminated in the recent worldwide approval of this therapeutic regimen for the management of bladder, esophageal, and lung cancer (1–3). Preclinical data emerging from investigator-initiated clinical trials of PDT of skin cancer are showing promising results (2, 3). This led to the approval of PDT for the treatment of actinic keratoses (2, 3). PDT of tumors begins with light activation of a photosensitizing compound localized in the malignant tissue to produce reactive oxygen species. In a typical protocol, a photosensitizer is administered systemically and is taken up by all tissues but is retained for longer times in cancerous and quickly proliferating cells. The photosensitizer with which we have been working is the silicon phthalocyanine Pc 4 [$HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$].

Studies from this and other laboratories have demonstrated the involvement of apoptosis as an early event in PDT-mediated killing of tumor cells *in vitro* (4) and ablation of tumors *in vivo* (2, 5, 6). Furthermore, we have shown that Pc 4 PDT results in WAF1/CIP1/p21-mediated G_0-G_1 arrest, followed by apoptosis in human epidermoid carcinoma cells A431 (7). More recently, we demonstrated the relevance of p21-mediated cell cycle deregulation and apoptotic tumor cell death under *in vivo* conditions (3, 8). The cascade of biomolecular events that lead to PDT-mediated cell killing is initiated by the damaging effects of reactive oxygen species, which are produced *in situ* as a result of photooxidative reactions during PDT (9). The generation of singlet oxygen is considered to play the central role in photodynamic cytotoxicity (9). Our understanding of the biomolecular mechanisms and components of PDT-mediated cytotoxicity is still far from being complete. Understanding these critical events during cell killing could lead to improvement in treatment protocols and enhance therapeutic efficacy. Clusterin, a widely expressed glycoprotein, also known as apolipoprotein J, has been shown to be highly up-regulated in regressing and involuting tissues responding to injury, cytotoxic chemicals, hormones, or developmental stimuli (10–12). A rapid induction of clusterin was demonstrated as an early event in various tissues undergoing apoptosis *in vitro* and *in vivo* (13–15). Similarly, enhanced expression of clusterin was found after exposure to high doses of tumor necrosis factor-α in a fibrosarcoma cell line with a constitutively low expression of clusterin (16). Clusterin transcript and protein levels were also highly induced by low levels of ionizing radiation (isolated as X-ray-induced transcript/protein-#8, xip8) in various human and rodent tumor cells (17, 18). Viard *et al.* (19) have demonstrated a significant increase of clusterin mRNA levels in human epidermoid carcinoma cells A431 in response to transient heat shock (20 min at 42°C) and the oxidative stress situations mediated via hydrogen peroxide, superoxide anion, hyperoxia, and UVA radiation. Here, we show the involvement of clusterin during Pc 4 PDT-mediated apoptosis *in vitro* as well as *in vivo* during the course of shrinkage of skin tumors.

Materials and Methods

Photosensitizer and Its Formulation. The water-insoluble silicon-phthalocyanine photosensitizer Pc 4 [$HOSiPcOSi(CH_3)_2(CH_2)_3N(CH_3)_2$] was synthesized and obtained from Ying-Syi Li and Malcolm E. Kenney from the Department of Chemistry, Case Western Reserve University, as described earlier (7). For the *in vitro* experiments, Pc 4 was dissolved in dimethyl formamide at a concentration of 0.5 μM. The *in vivo* PDT required our standardized formulation (a stock solution containing 1 mg of drug/ml was made by dissolving Pc 4 in 5% Cremophor EL, 5% absolute ethanol, and 95% normal saline and stored at −26°C). Prior to injection, this stock solution was mixed with an equal volume of 5% Cremophor EL, 5% ethanol, and 90% saline. The volume of injected solution was adjusted to give the desired dose in mg/kg body weight.

Cells. Human epidermoid carcinoma cells A431 were maintained in DMEM, whereas radiation-induced fibrosarcoma cells (RIF-1) were grown in Eagle's MEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and kept in an atmosphere of 95% air/5% CO_2 in a humidified incubator at 37°C. The experiments were performed on 70–80% confluent cells grown in 100 × 20-mm Falcon disposable cell culture dishes. Human breast cancer cells MCF-7:WS8 were maintained and plated for apolipoprotein J/xip8 induction as described previously (18).

***In Vitro* PDT.** A431 and RIF-1 cells were treated with 0.5 μM Pc 4 in complete DMEM overnight. Prior to light application, the photosensitizer-

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[3] The abbreviations used are: PDT, photodynamic therapy; Pc 4, phthalocyanine 4; PARP, poly(ADP-ribose) polymerase.

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containing media were aspirated and replaced with HBSS with Ca^{2+} and Mg^{2+}. Subsequently, cells were exposed to 20 kJ/cm^2 of light at 675 nm delivered using a 300-W halogen lamp. After irradiation, cells were incubated at 37°C in DMEM (complete media) for selected time points (1, 3, 6, 9, and 12 h after PDT). MCF-7 cells were irradiated with 10 Gy and subsequently incubated at 37°C in complete media. The cells were then harvested at 72 h after ionizing radiation.

Preparation of Cell Lysates. At desired times after the light treatment, media were aspirated, cells were washed with PBS [10 mM (pH 7.4)], and 0.5 ml of ice-cold lysis buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na_3VO_4, 0.5% NP40, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin (pH 7.4)] was added to each plate. Culture dishes were then placed on ice for 30 min. Cells were then scraped, the lysates were collected in Eppendorf microcentrifuge tubes and passed through a 21-gauge needle to break up cell aggregates. After centrifugation at 14,000 $\times$ *g* for 15 min at 4°C, supernatants (total cell lysates) were stored at −70°C. The protein content was estimated by DC Bio-Rad assay (Bio-Rad Laboratories, Hercules, CA) using the manufacturer's protocol.

Animals. Female SENCAR mice (obtained from the National Cancer Institute, Fredrick, MD) and female SKH-1 hairless mice (Charles River Laboratories, Wilmington, MA), both 6 weeks of age, were used for the *in vivo* study. The animals were subjected to a 12-h light/12-h dark cycle and housed in the Animal Resource Facility of Case Western Reserve University. To develop chemically induced cutaneous papillomas, the SENCAR mice were shaved with electric clippers, and Nair depilatory cream was applied. Forty-eight h later, the shaved area was treated with a single topical application of 7,12-dimethylbenz[*a*]anthracene (20 μg in 0.2 ml acetone/mouse). One week later, the animals were treated with the topical administration of 12-*O*-tetradecanoylphorbol-13-acetate two times per week (2.5 μg in 0.2 ml acetone/mouse). At 14 weeks using this protocol, the SENCAR mice presented with three to four benign squamous papillomas of 3–6-mm diameter. The SKH 1 mice were irradiated with UVB light at 180 mJ/cm^2 three times per week. Sixteen weeks later, these animals presented with small (2–4 mm), four to six squamous papillomas on the treated area.

***In Vivo* PDT.** For these studies, mice bearing four to six tumors were selected, and 48 h prior to irradiation, Pc 4 was injected i.v. at a dose of 1 mg/kg body weight. Two tumors per animal were irradiated with light at 672 nm, 150 J/cm^2, and 150 mW, delivered by a diode laser (Applied Optronics Corp., Newport, CT). Two lesions in the same animal were covered with a light-impermeable tape and were not exposed to laser light. These tumors served as controls. In prior experiments, we established that tumors treated this way exhibit no regression and had no effect on apoptotic promoters determined (6). At 1, 3, 6, 12, 24, and 48 h after PDT, the mice were sacrificed, and PDT-treated and untreated tumors were removed, immediately frozen in liquid nitrogen, and kept at −70°C.

Preparation of Tumor Cell Lysates. The tissues were homogenized in ice-cold lysis buffer (formulated as described above) and kept on ice for 30 min. After subsequent centrifugation at 14,000 $\times$ *g* for 15 min at 4°C, supernatants (total tumor cell lysates) were stored at −70°C. The protein concentration was estimated by DC Bio-Rad assay according to the manufacturer's protocol.

Western Blot Analysis. For Western blot analyses, 25–50 μg of protein were resolved on 10–12% polyacrylamide-SDS gels and transferred onto a nitrocellulose membrane as described (7). The membrane was blocked in blocking buffer (5% nonfat dry milk/1% Tween 20 in 20 mM TBS, pH 7.5) for 1.5 h at room temperature and incubated with the appropriate primary antibody (anti-SP-40,40 antibody, monoclonal, human reactive; Quidel Corp., San Diego, CA; anti-clusterin-antibody, polyclonal, reactive with clusterin of murine origin; Santa Cruz Biotechnology, Santa Cruz, CA) solved in blocking buffer overnight at 4°C, followed by incubation with complementary secondary antibody. Proteins were detected by chemiluminescence (ECL; Amersham International, Piscataway, NJ) and autoradiography using Hyperfilm ECL (Amersham International). In whole-cell extracts, two major protein forms were observed at M_r ~60,000–70,000 and another band at M_r ~40,000 (M_r ~80,000 when 3-mercaptoethanol was removed from the protein extraction buffer).

Results and Discussion

Numerous clinical studies have shown that PDT represents an effective and safe therapeutic modality for a variety of precancerous and malignant conditions (1–3). It is increasingly appreciated that apoptosis is involved in PDT-induced tumor cell kill *in vitro* (4) and tumor ablation *in vivo* (2, 5–6). The initial demonstration by Agarwal *et al.* (4) in 1991 that *in vitro* PDT results in a rapid induction of apoptosis and the subsequent reports from our and other laboratories established the apoptotic response to *in vivo* PDT (1–2, 5–6). This has raised the possibility that therapeutic response to PDT could be enhanced by understanding apoptotic pathways that occur during tumor shrinkage, which is the goal of this treatment modality. Characteristic biochemical markers of apoptosis have been observed 30 min after phthalocyanine- and porphyrin-mediated photodamage (2, 4–6). Because rapid induction of clusterin has been demonstrated in various tissues undergoing apoptosis *in vitro* and *in vivo* (13–15), we hypothesized a role for clusterin in PDT-induced apoptosis.

The presence of clusterin in our samples was verified by a comparison with the up-regulated clusterin protein in ionizing radiation-treated human breast cancer cells MCF-7 (Fig. 1), which are known for their induced clusterin levels after ionizing radiation treatment (18). Clusterin levels were measured in A431 cells at different time points (1, 3, 6, 9, and 12 h) after Pc 4-mediated photodynamic treatment. The PDT of these cells resulted in a significant up-regulation of the clusterin protein expression, as compared with untreated controls that showed only moderate clusterin levels, detected by Western blot analysis using the anti-SP-40,40 antibody (Fig. 2*A*). Because Pc 4 alone or light alone did not exert any effect on the expression of this protein, it is evident that the up-regulation observed is a response to PDT. The elevated clusterin levels in PDT-treated cells were visible as early as 1 h after the treatment. Cleavage of the nuclear enzyme PARP, an indicator of apoptotic cell death, was detected via Western blot analyses, demonstrating apoptosis 1 h after PDT, with maximal PARP cleavage 12 h after photodynamic treatment (Fig. 2*A*). The up-regulation of clusterin showed a time-dependent pattern reaching maximum levels at 12 h after PDT. The densitometric analyses of the clusterin protein bands and the cleaved PARP protein bands are shown in Fig. 2*B*. To support our hypothesis that clusterin expression may be linked to apoptosis, we investigated the clusterin protein expression in Pc 4 PDT-treated radiation-induced fibrosarcoma (RIF-1) cells, which do not undergo apoptosis in response to PDT (5). The clusterin levels in these cells remained unchanged at 0.5–6 h after PDT as compared with the untreated controls (Fig. 2*A*). To confirm the validity of the *in vitro* data,

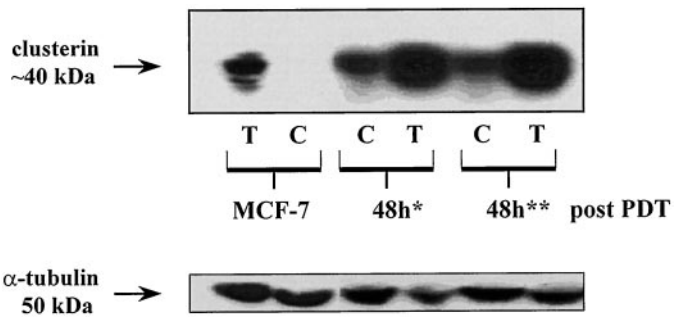

Fig. 1. Clusterin induction in MCF-7 cells attributable to treatment with ionizing radiation (*Lanes 1* and *2*). The cells were irradiated with 10 Gy, harvested, and extracted for clusterin and α-tubulin 72 h after treatment. *C*, untreated control; *T*, treated cells. *Lanes 3* and *4* demonstrate clusterin protein levels in chemically-induced murine skin papillomas (*) 48 h after Pc 4-mediated PDT in the tumor (*T*) and the corresponding control (*C*). *Lanes 5* and *6* show the clusterin expression in UVB-induced mouse papillomas (**) 48 h after PDT in the treated (*T*) and untreated (*C*) tumors.

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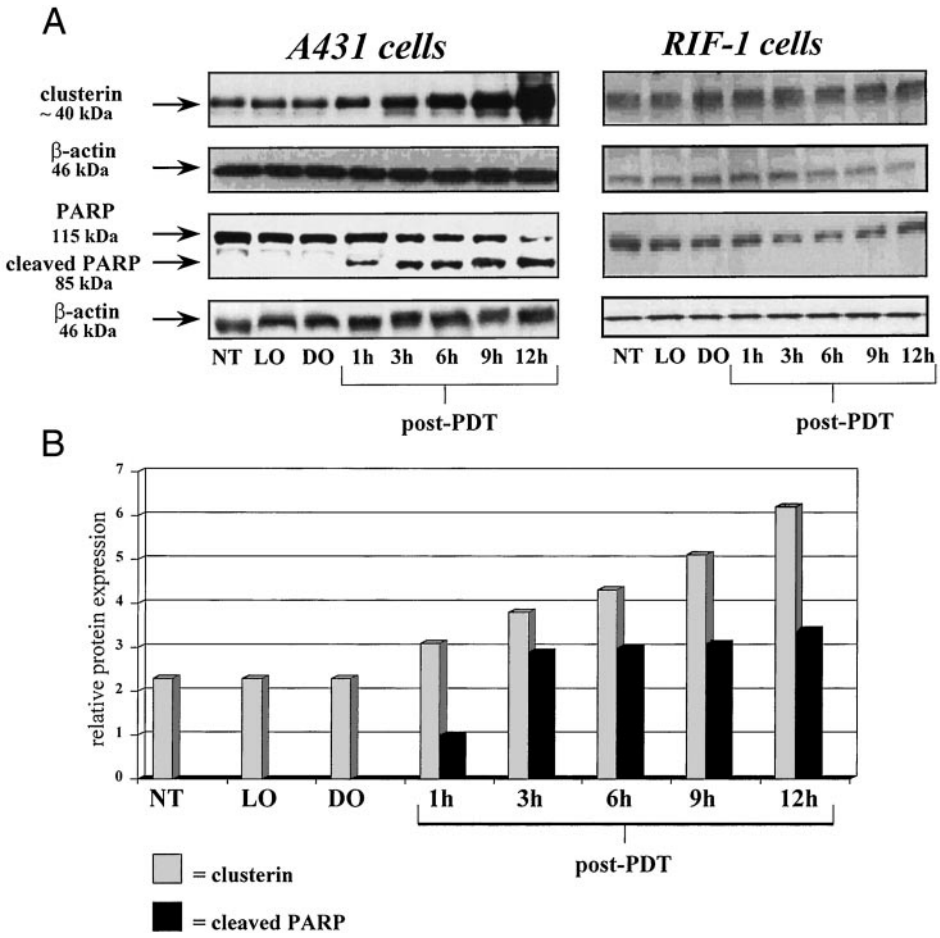

Fig. 2. *A*, Western blot analysis showing clusterin protein levels and PARP cleavage in response to Pc 4-mediated PDT of human epidermoid carcinoma A431 cells and radiation-induced fibrosarcoma (RIF-1) cells. All cells were irradiated except not treated controls (*NT, Lane 1*), and cells treated with drug only (*DO, Lane 3*). The cells in *Lane 2* received light only (*LO*). The cells in *Lanes 4–8* were subjected to PDT and harvested at different time points after the treatment. The analysis for β-actin demonstrates that the total protein content is equal in all lanes, confirming the accuracy of the protein estimation and the loading of the samples during Western blot procedure. *B*, densitometric quantification of clusterin induction and PARP cleavage with respect to β-actin levels in A431 cells at different time points after PDT, reflecting the results of the Western blot analysis. Cells were treated with PDT and then extracted for clusterin, PARP, and β-actin as described in "Materials and Methods."

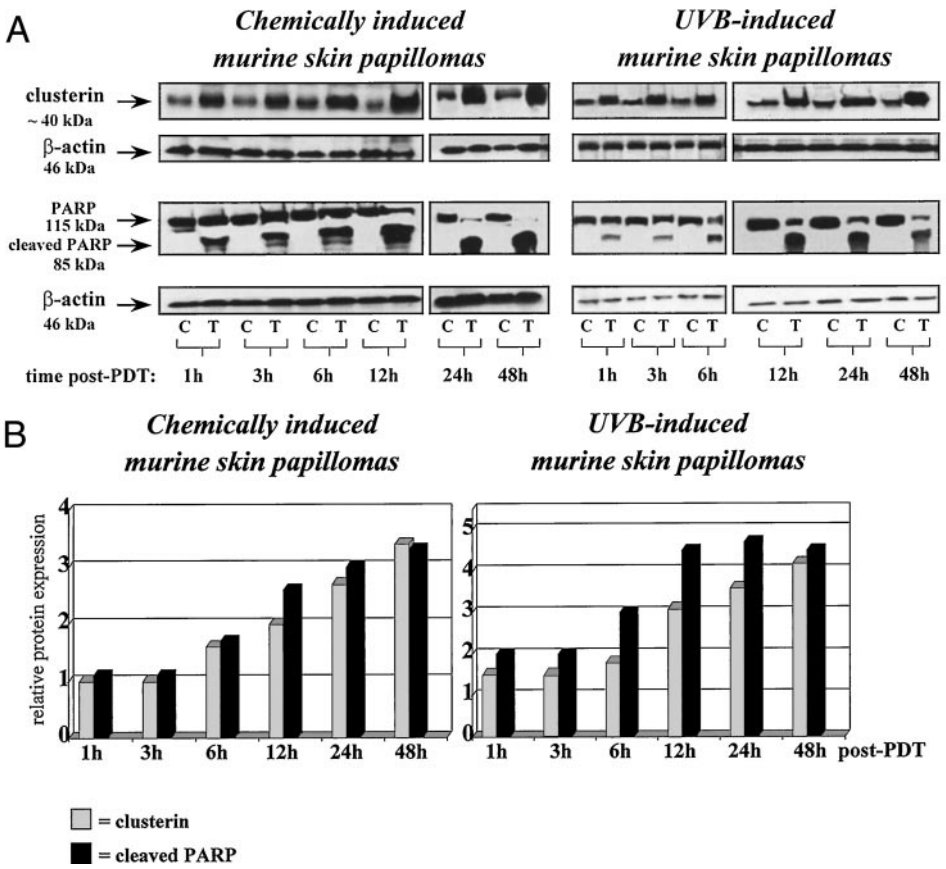

Fig. 3. *A*, Western blot analysis for clusterin expression, PARP cleavage, and β-actin control in chemically induced and UVB-induced murine skin tumors at different time points after PDT (*T*, tumor; *C*, control). Untreated control tumors were harvested at each corresponding time point from the same animal. *B*, densitometry of the clusterin protein bands and cleaved PARP protein bands in PDT-treated murine skin papillomas at different time points after PDT.

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clusterin protein levels were evaluated *in vivo*, using two different, PDT-treated murine squamous papilloma tumor models. As for *in vitro* experiments, clusterin and PARP levels were monitored. The macroscopic response to Pc 4 PDT in the form of pronounced tumor shrinkage and ablation occurred promptly 12–24 h after irradiation in both tumor groups, in the chemically as well as the UVB-induced skin papillomas. The Pc 4-based photodynamic treatment of chemically induced murine skin papillomas caused a time-dependent up-regulation of clusterin levels, with a significant increase observed as early as 1 h after PDT, peaking at the 24-h time point (Fig. 3*A*). Consistent with the *in vitro* situation in A431 cells, moderate clusterin levels were found in all untreated tumors. The results obtained by Western blot analyses of the UVB-induced skin papillomas were in accordance with the *in vitro* as well as the *in vivo* data described above. Pc 4 PDT induced clusterin protein expression in these tumors in a time-dependent manner as compared with untreated controls, starting 1 h after light administration and proceeding gradually, with maximal increase 48 h after PDT (Fig. 3*A*). PARP cleavage was observed as early as 1 h after the treatment in both chemically as well as UVB-induced tumors, reaching maximal levels at 24 h after PDT (Fig. 3*A*). Fig. 3*B* demonstrates the densitometry of the clusterin protein bands and cleaved PARP protein bands in the treated tumors.

These data, for the first time, demonstrate that clusterin is involved in the biochemical tumor response to photodynamic action during *in vitro* as well as *in vivo* PDT. On the basis of our previous observations that Pc 4 PDT leads to tumor ablation via induction of apoptosis, the present results suggest that clusterin may play a significant role in PDT-mediated apoptotic cell death. The exact mechanisms by which clusterin may regulate or contribute to the initiation of biochemical pathways that eventually trigger apoptosis, remain to be investigated in detail. According to a report by Bettuzzi *et al.* (15), the accumulation of clusterin-mRNA precedes the appearance of DNA degradation, starting to increase as early as 30 min after dexamethasone-induced cell death. Our experiments show that the up-regulation of clusterin protein expression occurs simultaneously with the induction of apoptosis during Pc 4-mediated photodamage, detectable 1 h after PDT. Thus, it is not clear whether clusterin plays an active part in apoptosis induction or merely serves as a marker of PDT-induced cell killing. Recent studies report the involvement of clusterin in response to heat shock and oxidative stress in A431 cells, demonstrating a rapid up-regulation of the protein within the first 40 min after exposure to heat shock (19). In contrast, A431 cells exposed to oxidative stress in the form of H_2O_2 or superoxide anions responded by a delayed increase of clusterin mRNA levels detectable 4 h after exposure, reaching maximum levels at 24 h after H_2O_2 addition (19). Considering the fact that the efficacy of photodynamic action is mainly based on the generation of reactive oxygen species via distinct photooxidative reactions, the induction of clusterin during PDT may be at least partly attributable to the oxidative stress induced in tumor cells by photosensitization. Further investigations will reveal the necessity of clusterin up-regulation for the induction of PDT-mediated apoptosis, as well as the correlation of clusterin with PDT-induced reactive oxygen species production. Because the findings presented in this report are based exclusively on Pc 4 PDT, it remains to be seen if this is a generalized effect of PDT with other photosensitizers or is exclusive to Pc 4 PDT. A better understanding of the involvement of clusterin in PDT-induced cell killing could lead to the development of new strategies to enhance the therapeutic efficacy of photodynamic treatment.

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