Published in final edited form as:

Lasers Med Sci. 2009 September ; 24(5): 777–786. doi:10.1007/s10103-008-0640-5.

Intracellular free calcium mediates glioma cell detachment and cytotoxicity after photodynamic therapy

Xin Hong,

Department of Neurosurgery, Henry Ford Hospital, Detroit, MI, USA

Feng Jiang,

Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

Steven N. Kalkanis,

Department of Neurosurgery, Henry Ford Hospital, Detroit, MI, USA

Zheng Gang Zhang,

Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

Xuepeng Zhang,

Department of Surgical Oncology, Affiliated Hospital, North China Coal Medical University, Tangshan City, Hebei Province, People's Republic of China

Xuguang Zheng,

Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

Hao Jiang, and

Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA

Michael Chopp

Department of Neurology, Henry Ford Hospital, 2799 West Grand Boulevard, Detroit, MI 48202, USA chopp@neuro.hfh.edu

Department of Physics, Oakland University, Rochester, MI, USA

Abstract

Photofrin photodynamic therapy (PDT) caused a dose-dependent decrease of enzymatic cell detachment by trypsin/ethylenediamine tetra-acetic acid (EDTA) in human glioma U251n and U87 cells. This happened coincidentally with the increase of intracellular free calcium ($[Ca^{2+}]_i$). Thapsigargin, which increased $[Ca^{2+}]_i$, induced further decrease in enzymatic cell detachment and increased cytotoxicity. Opposite effects were observed when 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetra-acetic acid tetrakis, an intracellular Ca^{2+} chelator, was used. PDT-induced changes in $[Ca^{2+}]_i$ and cell detachment were not blocked by calcium channel antagonists nickel (Ni^{2+}) or nimodipine, nor were they altered when cells were irradiated in a buffer free from Ca^{2+} and magnesium (Mg^{2+}), suggesting that $[Ca^{2+}]_i$ is derived from the internal calcium stores. Decreased cell migration was observed after PDT, as assessed by chemotactic and wound-healing assays. Our findings indicated that internal calcium store-derived $[Ca^{2+}]_i$ plays an important role in PDT-induced

enzymatic cell detachment decrease and cytotoxicity. Cell migration may be affected by these changes.

Keywords

Photodynamic therapy; Intracellular free calcium; Enzymatic cell detachment; Cytotoxicity

Introduction

Photodynamic therapy (PDT) is a promising anticancer treatment based on the destruction of photosensitizer-containing tumor cells induced by light. The mechanisms of PDT-induced tumor toxicity involve oxygen free radical or nitric oxide production, disturbance of intracellular free calcium $[Ca^{2+}]_i$ homeostasis, induction of apoptosis and activation of phospholipase [1–4]. After PDT, cultured cells exhibit increased attachment to the cell culture plastic substratum, and a longer digestion time is needed to detach these cells by trypsin/ethylenediamine tetra acetic acid (EDTA). The decreased cell detachment after PDT occurs when a variety of photosensitizers is used, including photofrin, aminolevulinic acid (ALA), hematoporphyrin derivatives (HPDs), benzoporphyrin derivative monoacid ring A (BPD-MA) and meta-tetra-hydroxyphenyl chlorine (mTHPC) [5–9]. This change in detachment may be important for cancer therapy, since cell adhesion and detachment play important roles in the remodeling of extracellular matrix, tumor invasion and formation of remote cancer metastases [10,11].

Cell adhesion provides a tight connection between cultured cells and the extracellular matrix (ECM). Over 50 proteins have been reported to be associated with focal contacts and are related to ECM adhesion. Among these, the integrin family contains the major transmembrane ECM receptors linking the extracellular matrix to the actin cytoskeleton [12–15]. Enzymatic cell detachment with trypsin/EDTA is a routine cell culture procedure in which trypsin is used to destroy adhesion molecules, such as integrins, between cell-to-cell and cell-to-ECM connections. EDTA is used to chelate Ca^{2+} , which is needed for calcium-dependent cell adhesion molecules (CAMs). Although PDT has been found to decrease cell detachment, little is known about how this happens. It has been reported that actin and microtubular cytoskeletal changes are related to decreased detachment in ALA-induced protoporphyrin IX-PDT, even with a sub-lethal ALA-PDT dose [6]. However, the linkage between PDT and the actin cytoskeleton remains unclear.

In this study we investigated if $[Ca^{2+}]_i$ was associated with PDT-induced enzymatic detachment change and cytotoxicity in human glioma cell lines U251n and U87, which differ in migration and proliferation. We also wanted to determine if increased $[Ca^{2+}]_i$ is derived from calcium influx or is released from the internal stores, and to examine whether cell migration is affected in these events after PDT.

Materials and methods

Chemicals and reagents

Photofrin (porfimer sodium) was manufactured by Lederle Parenterals, Inc. (Carolina, PR, USA) and was freshly prepared before experiments. Nickel chloride ($NiCl_2$), nimodipine, thapsigargin (TG), 1,2-bis(2-aminophenoxy) ethane-*N,N,N',N'*-tetra-acetic acid tetrakis (acetoxymethylester) (BAPTA-AM), digitonin, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA), sulforhodamine B and dimethylsulfoxide (DMSO) were obtained from Sigma (St. Louis, MO, USA). Fura-2 acetoxymethyl (AM) ester (Fura-2/AM) was purchased from Molecular Probes (Eugene, OR, USA).

Cell culture and laser exposure

U251n and U87 glioma cell lines were originally obtained from the American Type Culture Collection (ATCC). Cells were cultured and maintained at 37°C with 5% carbon dioxide (CO₂) in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS), 4 mM glutamine, 100 IU/ml penicillin, 100 µg/ml streptomycin, and 1% nonessential amino acid (Invitrogen).

U251n and U87 cells (5×10⁵ cells per well) were plated in six-well plates (Costar, Corning, NY, USA) and treated with photofrin overnight. Before laser exposure, the cell culture medium was replaced with 3 ml Locke's buffer [154 mM sodium chloride (NaCl), 5.6 mM potassium chloride (KCl), 3.6 mM sodium bicarbonate (NaHCO₃), 5.6 mM glucose, 5 mM 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid (HEPES), 1 mM magnesium chloride (MgCl₂), and 2.2 mM calcium chloride (CaCl₂), pH 7.2] [16,17]. The cells were then exposed to laser using a semiconductor diode laser system (University Health Network, Toronto, Canada). This system provided light (632 nm) of 6 mW/cm² irradiance at the position of the cells. For cytotoxicity detection, supernatants were changed with phenol red-free and serum-free DMEM after laser exposure. Two millimoles per liter of Ni²⁺, 10 µM of nimodipine or 100 µM of BAPTA-AM was added to the medium 1 h before laser irradiation.

PDT-induced cytotoxicity

Sulforhodamine B (SRB) assay was used to determine PDT-induced cytotoxicity [18]. Twenty four hours and 48 h after PDT, the cells were fixed with ice-cold 10% trichloro-acetic acid for 1 h at 4°C and then rinsed with water to remove trichloro-acetic acid. Two milliliters of 0.4% SRB dissolved in 1% acetic acid was added to each well and left for 30 min. SRB was removed, and the cells were washed in 1% acetic acid and allowed to dry in the air. Four milliliters of 10 mM hydroxymethyl aminomethane (Tris) was added to each well to solubilize the cell-bound dye, and the absorbance was measured at 540 nm. The results were expressed as a percentage of control cells.

PDT-induced cytotoxicity was also assessed by release of lactate dehydrogenase (LDH) from damaged cells into the medium, using a cytotoxicity detection kit (Roche Applied Science, Indianapolis, IN, USA), according to the manufacturer's instructions, with minor modifications. Twenty-four hours after PDT, the supernatants were collected and centrifuged at 10,000 r.p.m. for 5 min to remove floating cells and cell debris. After sedimentation, 100 µl of supernatant from the samples was transferred to a 96-well, flat-bottomed plate (Costar, Cambridge, MA, USA), to which 100 µl of substrate mix in assay buffer was then added. The plates were kept protected from light for up to 30 min at room temperature. The absorbance was monitored at 492 nm with the reference wavelength 620 nm in a plate reader (Multiskan MCC/340, Labsystems, Finland). The percentage of cytotoxicity produced by PDT was calculated relative to absorbance values for blank medium controls and values resulting from total lysis of cells by Triton X-100 (100% cell kill), according to the following formula: Cytotoxicity (%) = (OD_{treated cells} - OD_{blank control}) / (OD_{total cells} - OD_{blank control}) × 100.

Cell detachment assay

To keep our experimental conditions consistent, we performed all laser treatment and enzymatic detachment in Costar six-well plates, although there were no differences when other brand products with different surface chemistry were used [6]. U251n and U87 cells were plated at 5×10⁵ cells per well. Cells were almost confluent the next day. After being loaded with photofrin (1–16 µg/ml) overnight, all culture media were replaced with Locke's buffer, and the samples were exposed to laser light. After laser exposure, the buffer in the wells was removed. Cells were washed with Ca²⁺-free Mg²⁺-free phosphate-buffered saline (PBS) solution, and 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA, USA) was added to each well.

The plates were shaken slowly until cells were detached. Two milliliters of FBS-containing medium was then added into each well to stop the proteolytic action of trypsin. The number of detached cells was counted with a hemacytometer (American Optical, Buffalo, NY, USA). The duration of trypsinization was determined visually in each experiment until the cells in any well of the plate were firstly detached. In order to reduce variations between the experiments, we quantified the effect of photosensitization as the ratio N/N_0 , where N and N_0 were the numbers of detached cells in the photosensitized and control samples, respectively. For the experiments with BAPTA-AM treatment, the results were expressed as the ratio to control samples in the BAPTA-AM group (N_b).

Determination of intracellular free calcium using fura-2/AM assay

The cytoplasmic $[Ca^{2+}]_i$ was measured as described by others, with some minor modifications [19,20]. Briefly, the cells were loaded with 2 μM (final concentration) of fura-2/AM and incubated at 37°C for 30 min. Cell culture supernatants were then replaced with Locke's buffer. After laser treatment, the cells were detached with 0.05% trypsin and re-suspended in 1 ml Locke's buffer. The cell suspension of 200 μl (2×10^5 U251n cells and 1.6×10^5 U87 cells) was transferred to a 96-well fluorescence reading plate. The $[Ca^{2+}]_i$ was calculated spectrofluorometrically by the equation $[Ca^{2+}]_i = k_d \beta (R - R_{min}) / (R_{max} - R)$, where β is the ratio of $^{380}F_{max}$ (fluorescence intensity exciting at 380 nm for zero level of free Ca^{2+}) to $^{380}F_{min}$ (fluorescence intensity exciting at 380 nm for saturating level of free Ca^{2+}). The determination of fluorescence ratio (R) was performed with a fluorometer at two excitation wavelengths, 335 nm and 380 nm, with emissions measured at 515 nm. The maximal (R_{max}) and minimal (R_{min}) ratios were determined with 50 μM digitonin (Sigma) and 10 mM EGTA (Sigma), respectively. We determined the cell-specific dissociation constant (K_d) experimentally, using the standards of the calcium calibration buffer kit with magnesium (Molecular Probes).

Cell migration assay

We used a Chemicon QCM™ 96-well migration assay kit to study cell chemotactic migration. After being exposed to laser, the cells were detached by trypsin/EDTA. The trypsin reaction was stopped by the addition of FBS containing medium, and the cells were then re-suspended with serum-free medium. Fifty thousand cells were seeded in the upper chamber of the plate. DMEM with 10% FBS was added to the lower chamber as a chemoattractant. After 6 h of incubation, the cells migrated through the 8- μm pore size membrane, were detached, and were treated with lysis buffer/dye solution supplied with the kit. Aliquots of mixtures were read with a fluorescence reader with a 485/525 nm filter set. When seeding cells to the upper chamber of the plate, we prepared the same amounts of cells (5×10^4) and added them directly to the lysis buffer. The fluorescence values from these cells were used as total cell control. Cell migration was expressed as the percentage of fluorescence from the migrated cell to total cell fluorescence ($F_{migrated\ cells} / F_{total\ cells} \times 100$). The experiments were repeated three times with at least four duplicates of each treatment.

We also measured cell motility, using an in vitro wound-healing assay. Cells were seeded on six-well tissue culture plates and grown to confluence. After the cells had been exposed to laser, we created wounds by scraping monolayer cells with a 1 ml sterile pipette tip. The wounded mono-layers were washed twice with serum-free media to remove cell debris. Monolayers were incubated in cell culture medium, and the width of the wound was checked through a microscope.

Statistical analysis

Data were presented as mean $\pm$ standard deviation (SD) of separate experiments. Each experiment was repeated at least three times. Statistical significance was compared by one-

way analysis of variance (ANOVA). *P* values less than or equal to 0.05 between the two treatments were considered to be statistically significant.

Results

Cytotoxicity and decrease of cell detachment induced by PDT

PDT-induced cytotoxicity was detected by SRB and LDH release assays. Twenty-four hours after PDT, survival of U251n and U87 cells had decreased in a dose-dependent manner with increasing photofrin concentration. Cells treated with higher radiant exposure (i.e., 360 mJ/cm²) exhibited more cytotoxic effects than cells treated at 180 mJ/cm². Laser-treated cells became round and enlarged 24 h after PDT. Fewer than 5% of cells were damaged by a photofrin dose of 4 µg/ml. U87 cells showed approximately 10% higher cytotoxicity than U251n cells (Fig. 1a). Similar results were observed with LDH release assay. No further cytotoxic effects were observed 48 h after PDT as determined by LDH release assay. Those results indicated the acute cytotoxic effects of PDT on glioma cells (Fig. 1b).

U251n and U87 cells exhibited decreased cell detachment after laser irradiation, even at the sub-lethal photofrin dose of 4 µg/ml. The decreased cell detachment occurred within 5 min after laser irradiation and slowly recovered. One hour after PDT, decreased cell detachment had disappeared in the cells treated with 1 µg/ml and 2 µg/ml photofrin. Six hours after PDT, cell detachment only persisted in the group of U251n cells treated with 16 µg/ml photofrin (Fig. 2).

[Ca²⁺]_i mediates cell detachment and cytotoxicity after PDT

[Ca²⁺]_i was detected by fura-2/AM fluorescence assay at different time points after PDT. To avoid the chelating effect of EDTA on calcium, we detached the cells with 0.025% trypsin in Locke's buffer. First, we determined the calcium dissociation constant (*K_d*) of the two cell lines, using the standards of the calcium calibration buffer kit with calcium concentrations ranging from 0 to 39 µM. The *K_d* was 238 nM for U251n cells and 175 nM for U87 cells. [Ca²⁺]_i was determined under the same conditions as for the calibration kit. The results showed that [Ca²⁺]_i in U251n and U87 cells was approximately 10⁻⁷ M, and [Ca²⁺]_i in U251n cells was higher than that of U87 cells. After laser exposure (i.e., 2–16 µg/ml photofrin treatment), [Ca²⁺]_i in U251n and U87 cells was immediately increased. The increased [Ca²⁺]_i slowly declined and returned to control levels after 6 h (Fig. 3). As [Ca²⁺]_i and cell detachment change showed the same pattern after PDT, we theorized that [Ca²⁺]_i fluctuation might be responsible for changes in cell detachment. To test this hypothesis, we used TG, which is an effective inhibitor of the Ca²⁺ ion pump in the endoplasmic reticulum (ER) [21–23]. Ten micromoles of TG induced an approximately two-fold increase of [Ca²⁺]_i in U251n and U87 cells. Pretreatment with TG 1 h before laser irradiation caused a higher level of [Ca²⁺]_i than photofrin treatment alone (*P*<0.01). Pretreatment with TG also caused increased cytotoxicity and further decreased cell detachment in both cell lines (*P*<0.01). In contrast, BAPTA-AM, an intracellular calcium chelator, blocked PDT-induced [Ca²⁺]_i increase. Cell detachment changes and cytotoxicity were also blocked by 100 µM of BAPTA-AM pretreatment. TG and BAPTA-AM pretreatment alone induced approximately 10% more LDH release than did the control, indicating the importance of [Ca²⁺]_i in cell survival (Fig. 4).

Ca²⁺ is released from internal stores after PDT

To determine the source of increased [Ca²⁺]_i by PDT, we used Ni²⁺ and nimodipine, L-type Ca²⁺ channel antagonists. Ten micrograms per milliliter nimodipine and 2 mM Ni²⁺ have been reported to inhibit Ca²⁺ influx effectively [24–26]. When we used the same doses, our results showed that nimodipine and Ni²⁺ did not block PDT-induced [Ca²⁺]_i increase (Fig. 5a). Additionally, nimodipine and Ni²⁺ did not block changes in cell detachment tested at 8 µg/ml

and 16 $\mu\text{g/ml}$ photofrin (Fig. 5b). Considering that Ca^{2+} influx may be mediated through ways other than voltage-dependent Ca^{2+} channels, we removed Ca^{2+} and Mg^{2+} from Locke's buffer when performing laser irradiation. We found that $[\text{Ca}^{2+}]_i$ and cell detachment exhibited no difference when compared with that in cells treated with Ca^{2+} and Mg^{2+} containing Locke's buffer (Fig. 5).

PDT at sub-lethal dose decreases glioma cell migration

To determine if decreased cell detachment was combined with cell migration change, we performed a cell chemotactic migration assay and a wound healing assay. In the chemotactic assay, cells were detached by trypsin/EDTA and loaded to the upper chamber of a chemotactic plate after laser treatment. The number of migrated cells decreased after 4 $\mu\text{g/ml}$ and 8 $\mu\text{g/ml}$ photofrin treatment ($P < 0.01$) (Fig. 6a). In the wound healing assay, a wider wound was observed after photofrin dosages of 4 $\mu\text{g/ml}$ and 8 $\mu\text{g/ml}$. The widths of the wounds were no different after photofrin dosages of 1 $\mu\text{g/ml}$ and 2 $\mu\text{g/ml}$ than were those of the control (i.e., no laser treatment) ($P < 0.01$) (Fig. 6b). As the migration of U251n cells was observed to be faster than that of the U87 cells in this assay, the end point was different when the wound width was measured. The migration time was 16 h for the U251n cells and 24 h for the U87 cells.

Discussion

Although U251n cells normally exhibit a higher growth rate than U87 cells, the results showed that U87 cells were a little more sensitive than U251n cells to photofrin PDT. As 180 mJ/cm² laser treatment caused less than 5% cell death in both U251n and U87 cells at a dose of 4 $\mu\text{g/ml}$ photofrin, we considered that 4 $\mu\text{g/ml}$ photofrin was a sub-lethal dose with the laser fluence used. After photofrin PDT, the cells showed a decrease in enzymatic detachment within 5 min after laser irradiation, even after a sub-lethal dose of photofrin. Such a rapid change is substantially shorter than the time required for gene expression or protein synthesis. Thus, photofrin likely changes the structure of molecules related to cell adhesion, and these structural changes may cause resistance of cells to enzymatic detachment or block enzyme access to focal complexes.

PDT-induced $[\text{Ca}^{2+}]_i$ increase has been well studied [27–30]. However, there is no direct evidence that $[\text{Ca}^{2+}]_i$ is correlated with cell detachment change. In our study we found that the rapid increase of $[\text{Ca}^{2+}]_i$ was coincidental with decreased cell detachment. This led us to speculate that increased $[\text{Ca}^{2+}]_i$ is involved in cell detachment change. We used TG and BAPTA-AM, which exhibited opposite effects in the regulation of $[\text{Ca}^{2+}]_i$, to obtain additional evidence on the role of $[\text{Ca}^{2+}]_i$ in cell detachment change and photofrin PDT-induced cytotoxicity. In addition to PDT-induced $[\text{Ca}^{2+}]_i$ increase, TG elevated $[\text{Ca}^{2+}]_i$ and caused a further decrease in cell detachment. TG also caused increased cytotoxicity in addition to photofrin PDT alone, while BAPTA-AM chelated intracellular calcium and greatly diminished PDT-induced cell detachment decrease and cytotoxicity. Our results clearly indicated that $[\text{Ca}^{2+}]_i$ plays an important role in cell behavior after photofrin PDT. We postulate that cell enzymatic detachment is an early and sensitive effect in response to increased $[\text{Ca}^{2+}]_i$. Although this increase seems to be reversible, $[\text{Ca}^{2+}]_i$ may lead to irreversible apoptosis or cell toxicity when exceeding certain levels [31–34].

Generally, it was thought that increased $[\text{Ca}^{2+}]_i$ after PDT was a result of Ca^{2+} influx [35, 36]. However, ALA-protoporphyrin IX, which remained in mitochondria and remote from the cell surface where adhesion occurs, also inhibited enzymatic detachment [37]. This observation indicates that increased $[\text{Ca}^{2+}]_i$ may come from sources other than the extra-cellular medium. In our study we inhibited Ca^{2+} influx using L-type voltage-dependent calcium channel antagonists. However, Ni^{2+} and nimodipine did not block increases in $[\text{Ca}^{2+}]_i$ and cell detachment changes. Similar results were found when Ca^{2+} and Mg^{2+} were removed from

Locke's buffer during laser irradiation. These data indicated that the increased $[Ca^{2+}]_i$ was not the result of Ca^{2+} influx but was derived from a release from internal Ca^{2+} stores. Thus, PDT-induced cell detachment changes may have been mainly due to the cells' resistance to trypsin reaction. It is possible that activated photofrin damaged the intracellular membrane system and released Ca^{2+} from mitochondria, ER, Golgi apparatus or nuclear calcium stores. Increased $[Ca^{2+}]_i$ may activate the actin cytoskeleton system to form a tight connection between cells and between cells and matrix, which blocks the access of trypsin to the focal complex.

As discussed above, $[Ca^{2+}]_i$ is an important factor in photofrin PDT-induced cell physiological behaviors. Next, we want to investigate if $[Ca^{2+}]_i$ or cell detachment changes affect the ability of cells to migrate. Evaluated by cell chemotactic migration and wound healing assays, decreased cell migration was observed at photofrin doses of 4 $\mu\text{g/ml}$ or higher. Although decreased cell migration at a dose of 8 $\mu\text{g/ml}$ photofrin may be explained by cell damage from PDT, a dose of 4 $\mu\text{g/ml}$ photofrin, which is a sub-lethal dose, also significantly decreased cell migration, indicating that $[Ca^{2+}]_i$ or cell detachment changes may be involved in migration regulation to some extent.

In conclusion, an increase in $[Ca^{2+}]_i$ is an important molecular event in photofrin PDT-induced inhibition of cell detachment and cytotoxicity. The increased $[Ca^{2+}]_i$ may be caused by Ca^{2+} released from internal stores. PDT-induced cell detachment changes may affect cell migration and facilitate tumor therapy. However, the mechanisms underlying the effects of activated photosensitizer on $[Ca^{2+}]_i$ release, Ca^{2+} on cell adhesion structure, and related signal transduction processes after photofrin PDT, warrant further investigation.

Acknowledgments

This work was supported by National Institutes of Health (NIH) grants PO1 CA043892 and RO1 CA100486. We thank Kevin Nelson for his technical assistance.

References

1. Yamamoto F, Ohgari Y, Yamaki N, Kitajima S, Shimokawa O, Matsui H, Taketani S. The role of nitric oxide in delta-aminolevulinic acid (ALA)-induced photosensitivity of cancerous cells. *Biochem Biophys Res Commun* 2007;353:541–546. [PubMed: 17196160]doi:10.1016/j.bbrc.2006.12.007
2. Lu Z, Tao Y, Zhou Z, Zhang J, Li C, Ou L, Zhao B. Mitochondrial reactive oxygen species and nitric oxide-mediated cancer cell apoptosis in 2-butylamino-2-demethoxyhypocrellin B photodynamic treatment. *Free Radic Biol Med* 2006;41:1590–1605. [PubMed: 17045927] doi:10.1016/j.freeradbiomed.2006.08.021
3. Tajiri H, Hayakawa A, Matsumoto Y, Yokoyama I, Yoshida S. Changes in intracellular Ca^{2+} concentrations related to PDT-induced apoptosis in photosensitized human cancer cells. *Cancer Lett* 1998;128:205–210. [PubMed: 9683284]doi:10.1016/S0304-3835(98)00066-4
4. Hendrickx N, Dewaele M, Buytaert E, Marsboom G, Janssens S, Van Boven M, Vandenheede JR, de Witte P, Agostinis P. Targeted inhibition of p38 α MAPK suppresses tumor-associated endothelial cell migration in response to hypericin-based photodynamic therapy. *Biochem Biophys Res Commun* 2005;337:928–935. [PubMed: 16214108]doi:10.1016/j.bbrc.2005.09.135
5. de Vree WJ, Fontijne-Dorsman AN, Koster JF, Sluiter W. Photodynamic treatment of human endothelial cells promotes the adherence of neutrophils in vitro. *Br J Cancer* 1996;73:1335–1340. [PubMed: 8645576]
6. Uzdensky A, Kolpakova E, Juzeniene A, Juzenas P, Moan J. The effect of sub-lethal ALA-PDT on the cytoskeleton and adhesion of cultured human cancer cells. *Biochim Biophys Acta* 2005;1722:43–50. [PubMed: 15716135]
7. Foultier MT, Vonarx-Coinsmann V, Cordel S, Combret A, Patrice T. Modulation of colonic cancer cell adhesiveness by haematoporphyrin derivative photodynamic therapy. *J Photochem Photobiol B* 1994;23:9–17. [PubMed: 8021754]doi:10.1016/1011-1344(93)06975-9

8. Ball DJ, Mayhew S, Vernon DI, Griffin M, Brown SB. Decreased efficiency of trypsinization of cells following photodynamic therapy: evaluation of a role for tissue transglutaminase. *Photochem Photobiol* 2001;73:47–53. [PubMed: 11202365]
doi:10.1562/0031-8655(2001)073<0047:DEOTOC>2.0.CO;2
9. Margaron P, Sorrenti R, Levy JG. Photodynamic therapy inhibits cell adhesion without altering integrin expression. *Biochim Biophys Acta* 1997;1359:200–210. [PubMed: 9434126]
doi:10.1016/S0167-4889(97)00115-8
10. Haier J, Nicolson GL. The role of tumor cell adhesion as an important factor in formation of distant colorectal metastasis. *Dis Colon Rectum* 2001;44:876–884. [PubMed: 11391152]
doi:10.1007/BF02234713
11. Kawaguchi T. Cancer metastasis: characterization and identification of the behavior of metastatic tumor cells and the cell adhesion molecules, including carbohydrates. *Curr Drug Targets Cardiovasc Haematol Disord* 2005;5:39–64. [PubMed: 15720223]doi:10.2174/1568006053005038
12. Gumbiner BM. Cell adhesion: the molecular basis of tissue architecture and morphogenesis. *Cell* 1996;84:345–357. [PubMed: 8608588]doi:10.1016/S0092-8674(00)81279-9
13. Watt FM. Role of integrins in regulating epidermal adhesion, growth and differentiation. *EMBO J* 2002;21:3919–3926. [PubMed: 12145193]doi:10.1093/emboj/cdf399
14. Zamir E, Geiger B. Molecular complexity and dynamics of cell-matrix adhesions. *J Cell Sci* 2001;114:3583–3590. [PubMed: 11707510]
15. Bhatt A, Kaverina I, Otey C, Huttenlocher A. Regulation of focal complex composition and disassembly by the calcium-dependent protease calpain. *J Cell Sci* 2002;115:3415–3425. [PubMed: 12154072]
16. Reichsman F, Santos S, Westhead EW. Two distinct ATP receptors activate calcium entry and internal calcium release in bovine chromaffin cells. *J Neurochem* 1995;65:2080–2086. [PubMed: 7595493]
17. Dow GS, Hudson TH, Vahey M, Koenig ML. The acute neurotoxicity of mefloquine may be mediated through a disruption of calcium homeostasis and ER function in vitro. *Malar J* 2003;2:14. [PubMed: 12848898]doi:10.1186/1475-2875-2-14
18. Haywood-Small SL, Vernon DI, Griffiths J, Schofield J, Brown SB. Phthalocyanine-mediated photodynamic therapy induces cell death and a G0/G1 cell cycle arrest in cervical cancer cells. *Biochem Biophys Res Commun* 2006;339:569–576. [PubMed: 16300726]
doi:10.1016/j.bbrc.2005.11.046
19. Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J Biol Chem* 1985;260:3440–3450. [PubMed: 3838314]
20. Malgaroli A, Milani D, Meldolesi J, Pozzan T. Fura-2 measurement of cytosolic free Ca²⁺ in monolayers and suspensions of various types of animal cells. *J Cell Biol* 1987;105:2145–2155. [PubMed: 3680375]doi:10.1083/jcb.105.5.2145
21. Baranska J, Chaban V, Czarny M, Sabala P. Changes in Ca²⁺ concentration in phorbol ester and thapsigargin treated glioma C6 cells. The role of protein kinase C in regulation of Ca²⁺ entry. *Cell Calcium* 1995;17:207–215. [PubMed: 7621533]doi:10.1016/0143-4160(95) 90035-7
22. Kijima Y, Ogunbunmi E, Fleischer S. Drug action of thapsigargin on the Ca²⁺ pump protein of sarcoplasmic reticulum. *J Biol Chem* 1991;266:22912–22918. [PubMed: 1835973]
23. Wenzel-Seifert K, Krautwurst D, Musgrave I, Seifert R. Thapsigargin activates univalent- and bivalent-cation entry in human neutrophils by a SK&F I3 96365- and Gd³⁺-sensitive pathway and is a partial secretagogue: involvement of pertussis-toxin-sensitive G-proteins and protein phosphatases 1/2A and 2B in the signal-transduction pathway. *Biochem J* 1996;314:679–686. [PubMed: 8670085]
24. Doroshenko N, Doroshenko P. The glutathione reductase inhibitor carmustine induces an influx of Ca²⁺ in PC12 cells. *Eur J Pharmacol* 2004;497:17–24. [PubMed: 15321730]
doi:10.1016/j.ejphar.2004.06.043
25. Tjong YW, Jian K, Li M, Chen M, Gao TM, Fung ML. Elevated endogenous nitric oxide increases Ca²⁺ flux via L-type Ca²⁺ channels by S-nitrosylation in rat hippocampal neurons during severe hypoxia and in vitro ischemia. *Free Radic Biol Med* 2007;42:52–63. [PubMed: 17157193]
doi:10.1016/j.freeradbiomed.2006.09.020

26. Xie L, Clunn GF, Lymn JS, Hughes AD. Role of intracellular calcium ($[Ca^{2+}]_i$) and tyrosine phosphorylation in adhesion of cultured vascular smooth muscle cells to fibrinogen. *Cardiovasc Res* 1998;39:475–484. [PubMed: 9798532]doi:10.1016/S0008-6363(98)00079-0
27. Hayashi M, Kanno T. Photodynamic stimulation causes sustained increase in intracellular calcium concentration in cells of small cell lung carcinoma. *Jpn J Vet Res* 1998;46:65–72. [PubMed: 10093416]
28. Ben-Hur E, Dubbelman TM, Van Steveninck J. Phthalocyanine-induced photodynamic changes of cytoplasmic free calcium in Chinese hamster cells. *Photochem Photobiol* 1991;54:163–166. [PubMed: 1780353]doi:10.1111/j.1751-1097.1991.tb02002.x
29. Hubmer A, Hermann A, Uberriegler K, Krammer B. Role of calcium in photodynamically induced cell damage of human fibroblasts. *Photochem Photobiol* 1996;64:211–215. [PubMed: 8787016]doi:10.1111/j.1751-1097.1996.tb02444.x
30. Zhou Z, Yang H, Zhang Z. Role of calcium in phototoxicity of 2-butylamino-2-demethoxy-hypocrellin A to human gastric cancer MGC-803 cells. *Biochim Biophys Acta* 2003;1593:191–200. [PubMed: 12581863]doi:10.1016/S0167-4889(02)00386-5
31. Zheng JH, Shi D, Zhao Y, Chen ZL. Role of calcium signal in apoptosis and protective mechanism of colon cancer cell line SW480 in response to 5-aminolevulinic acid-photodynamic therapy (in Chinese). *Ai Zheng* 2006;25:683–688. [PubMed: 16764761]
32. Inanami O, Yoshito A, Takahashi K, Hiraoka W, Kuwabara M. Effects of BAPTA-AM and forskolin on apoptosis and cytochrome c release in photosensitized Chinese hamster V79 cells. *Photochem Photobiol* 1999;70:650–655. [PubMed: 10546560]doi:10.1111/j.1751-1097.1999.tb08265.x
33. Ben-Hurt E, Dubbelman TMAR. Cytoplasmic free calcium changes as a trigger mechanism in the response of cells to photosensitization. *Photochem Photobiol* 1993;58:890–894. [PubMed: 8310012]doi:10.1111/j.1751-1097.1993.tb04989.x
34. Marks DI, Fox RM. Mechanisms of photochemotherapy-induced apoptotic cell death in lymphoid cells. *Biochem Cell Biol* 1991;69:754–760. [PubMed: 1799445]
35. Joshi PG, Joshi K, Mishra S, Joshi NB. Ca^{2+} influx induced by photodynamic action in human cerebral glioma (U-87 MG) cells: possible involvement of a calcium channel. *Photochem Photobiol* 1994;60:246–248. doi:10.1111/j.1751-1097.1994.tb05098.x
36. Specht KG, Rodgers MA. Plasma membrane depolarization and calcium influx during cell injury by photodynamic action. *Biochim Biophys Acta* 1991;1070:60–68. [PubMed: 1751539]doi:10.1016/0005-2736(91)90146-Y
37. Uzdensky A, Juzeniene A, Ma LW, Moan J. Photodynamic inhibition of enzymatic detachment of human cancer cells from a substratum. *Biochim Biophys Acta* 2004;1670:1–11. [PubMed: 14729136]

Fig. 1.

Photofrin PDT-induced cytotoxicity. Cells were pretreated with 1–16 $\mu\text{g/ml}$ photofrin overnight and then exposed to laser at 6 mW/cm^2 . Cytotoxicity was determined by SRB and LDH release assays. **a** The survival of U251n and U87 cells were determined by SRB assay 24 h after photofrin PDT. Each cell line was irradiated with laser at 180 mJ/cm^2 and 360 mJ/cm^2 , respectively. **b** LDH release in U251n and U87 cells was measured 24 h and 48 h after laser exposure at 180 mJ/cm^2 . ** $P < 0.01$ compared with no laser control cells

Fig. 2. Enzymatic cell detachment after photofrin PDT. U251n and U87 cells were detached by trypsin/EDTA 5 min, 1 h, 3 h and 6 h after 180mJ/cm^2 laser exposure. **a** Undetached cells were stained with crystal violet 5 min after photofrin PDT. **b** Numbers of detached cells were counted at different time points after photofrin PDT. Values represent the mean ratio of the number of photosensitized cells (N) to the number of untreated (no photofrin, not irradiated) cells (N_0)

Fig. 3.

Increased intracellular free calcium ($[Ca^{2+}]_i$) induced by photofrin PDT at 180 mJ/cm^2 laser irradiation. $[Ca^{2+}]_i$ was measured 5 min, 3 h and 6 h after laser exposure with different doses of photofrin by fura-2/AM fluorescence assay. $**P < 0.01$ compared with cells not exposed to laser in the group treated with the same photofrin dose

Fig. 4. Effects of TG and BAPTA-AM on $[Ca^{2+}]_i$, cell detachment and cytotoxicity of U251n and U87 cells after photofrin PDT at 180 mJ/cm^2 laser irradiation. **a** TG and BAPTA-AM pretreatment on PDT-induced $[Ca^{2+}]_i$ increase. $[Ca^{2+}]_i$ was determined 5 min after laser exposure. Values represent the mean ratio of the number of photosensitized cells (N) to the number of BAPTA-AM control cells (not treated with photofrin) (Nb). **b** TG and BAPTA-AM pretreatment on cell detachment change detected 5 min after laser exposure. **c** TG and BAPTA-AM pretreatment on PDT-induced cytotoxicity as determined 24 h after laser exposure by LDH release assay. * $P < 0.05$, ** $P < 0.01$ compared with the group treated with the same photofrin dose

Fig. 5.

Effect of extracellular Ca^{2+} on photofrin PDT-induced $[Ca^{2+}]_i$ increase and cell detachment change. U251n and U87 cells were pretreated with 2 mM Ni^{2+} or 10 μ M nimodipine 1 h before 180 mJ/cm² laser exposure. $[Ca^{2+}]_i$ levels (**a**) and cell detachment (**b**) were determined 5 min after photofrin PDT (detachment expressed as N/No, see legend of Fig. 2). The experiments were also performed with cells in Ca^{2+} -free Mg^{2+} -free Locke's buffer when laser irradiation was performed

Fig. 6.

Cell migration after photofrin PDT at 180 mJ/cm² laser exposure. **a** Cell migration was evaluated with a Chemicon chemotactic migration kit. Five minutes after laser exposure, cells were detached and 5×10^4 cells were loaded in the upper chamber of the migration plate. Cells Migrating towards 10% FBS were detected 6 h after PDT by fluorescence assay. Data are expressed as percentage of $F_{\text{migrated cells}}$ to $F_{\text{total cells}}$, as described in the Methods section. **b** Cell migration detected by wound healing assay after PDT. Five minutes after laser exposure, a wound was made as described in the Methods section. The width of the wound was measured 16 h later for U251n cells and 24 h later for U87 cells. $**P < 0.01$ compared with cells not exposed to laser in the group treated with the same dose of photofrin