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Photodynamic inhibition of enzymatic detachment of human cancer cells from a substratum

Anatoly Uzdensky[a,b,*], Asta Juzeniene[a], Li-Wei Ma[a], Johan Moan[a]

[a] *Institute for Cancer Research, 0310 Montebello, Oslo, Norway*
[b] *Rostov State University, 344090, Rostov-on-Don, Russia*

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Abstract

Photodynamic therapy (PDT) is currently used for cancer treatment. It is shown that sublethal PDT of human WiDr adenocarcinoma cells and D54Mg glioblastoma cells with 5-aminolevulinic acid (ALA), disulfonated tetraphenylporphyrine ($TPPS_{2a}$), or MitoTracker Red (MTR) inhibits their trypsin-induced detachment from a plastic substratum. $TPPS_{2a}$ was bound selectively to the plasma membrane, whereas MTR was found in mitochondria. Both granular and diffuse fluorescence of ALA-derived protoporphyrin IX (PpIX) was observed in the perinuclear cytoplasm but not in the plasma membrane of WiDr cells stained for 2 h with 1 mM ALA. In D54Mg cells, PpIX fluorescence was observed not only in the cytoplasm but also in the plasma membrane. Fluorescence measurements showed a progressive accumulation of PpIX in the WiDr cells during incubation with ALA and a PpIX efflux into the medium after 1 h or longer incubation. PpIX retained in the plasma membrane during efflux may be responsible for PDT-induced impairment of cell adhesion. On the other hand, MTR-PDT or ALA-PDT after 15-min incubation, when the newly synthesized PpIX should remain in mitochondria, also inhibited enzymatic cell detachment. Therefore, photodynamic targeting of mitochondria, remote from the cell surface where adhesion occurs, may disturb cell adhesion. Photodynamic inhibition of enzymatic cell detachment may be related to PDT-induced inhibition of tumour metastasis.

Keywords: ALA-PDT; MitoTracker Red; $TPPS_{2a}$; Cell adhesion; Photosensitizer localization; Detachment

1. Introduction

Photodynamic therapy (PDT) is based on light induced destruction of selectively stained pathological tissues in the presence of oxygen. Singlet oxygen and secondary free radicals generated by photoexcited dye molecules can destroy several cellular structures [1,2]. An important, but not well-studied effect of PDT is its influence on cell adhesion. Two aspects of photodynamic influence on cell adhesion processes have been described: (i) the effect on cell attachment to a plastic substratum [5,6], to a substratum coated with extracellular matrix proteins [7,8], or to other cells [5,6]. (ii) Effects on trypsin/EDTA-induced detachment of cultured cells from a substratum [5,9,10]. Hematoporphyrin derivatives (HPD) [5,6,9,10], benzoporphyrin derivative monoacid ring A (BPD-MA) [7,8], zinc phthalocyanine derivative and meso-tetra-hydroxyphenyl-chlorin (mTHPC) [10] were used as photosensitizers in these studies. These effects are important for cancer therapy since cell adhesion may play a significant role in cancer metastasis [11,12]. Photodynamic treatment has been found to reduce cancer metastases [13,14].

5-Aminolevulinic acid (ALA), a biochemical precursor of the potent endogenous photosensitizer protoporphyrin IX (PpIX), is being successfully used for cancer treatment [1,3,4]. PpIX accumulates in rapidly proliferating cancer cells, thus providing selective destruction of tumours. In recent years the mechanisms of ALA-PDT have been thoroughly studied on the cellular and tissue levels [3,4]. However, the effect of ALA-PDT on adhesive properties of cultured cancer cells has not been studied so far.

Abbreviations: ALA, 5-aminolevulinic acid; BPD-MA, benzoporphyrin derivative monoacid ring A; DMSO, dimethyl sulfoxide; EDTA, ethylenediaminetetraacetate; mTHPC, meso-tetra-hydroxyphenyl-chlorin; MTR, MitoTracker Red CMXRos; PBS, phosphate-buffered saline; PDT, photodynamic therapy; PpIX, protoporphyrin IX; $TPPS_{2a}$, disulfonated meso-tetraphenylporphyrine

* Corresponding author. Department of Biophysics, Rostov State University, 194/1 Stachy ave., NIINK, 344090, Rostov-on-Don, Russia. Tel.: +7-8632-433577; fax: +7-8632-433588.
E-mail address: uzd@krinc.ru (A. Uzdensky).

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The goal of the present work was to study the PDT effect on enzymatic detachment from a substratum of cells of two human cell lines differing in morphology and colony-forming behaviour: WiDr adenocarcinoma and D54Mg glioblastoma cells. Three photosensitizers were chosen: PpIX, endogenously produced from ALA; MitoTracker Red CMXRos (MTR), selectively staining mitochondria; and disulfonated meso-tetraphenylporphyrine ($TPPS_{2a}$), selectively staining the plasma membrane. It was shown that sublethal ALA-PDT as well as photodynamic targeting of mitochondria or the plasma membrane (using MTR or $TPPS_{2a}$, respectively) inhibit trypsin-induced cell detachment.

2. Materials and methods

2.1. Chemicals

ALA, dimethyl sulfoxide (DMSO) and trypsin were purchased from Sigma-Aldrich Norway AS (Oslo, Norway). Solution (30 mM) of ALA in RPMI-1640 medium (GIBCO, Grand Island, NY) was prepared ex tempera before every experiment. Then it was diluted in this medium to a final concentration of 1 mM. Mitotracker Red CMXRos (Molecular Probes, Eugene, OR) dissolved in DMSO (1 mM) was diluted in phosphate buffered saline (PBS) to a concentration of 50 μM, aliquoted and stored frozen at −20 °C. Its working concentration in RPMI 1640 medium was 200 nM. The stock solution of $TPPS_{2a}$ (Porphyrin Products, Logan, UT) in DMSO (0.45 mM) was diluted in RPMI 1640 medium to a final concentration of 0.9 μM.

2.2. Cell cultivation

The human WiDr cells derived from a primary adenocarcinoma of the rectosigmoid colon [15] were maintained in exponential growth in RPMI 1640 medium with 10% foetal calf serum (FCS), 100 units/ml penicillin and 100 mg/ml streptomycin. D54Mg glioblastoma cells [16] were grown in Dulbecco's modified Eagle medium with 10%

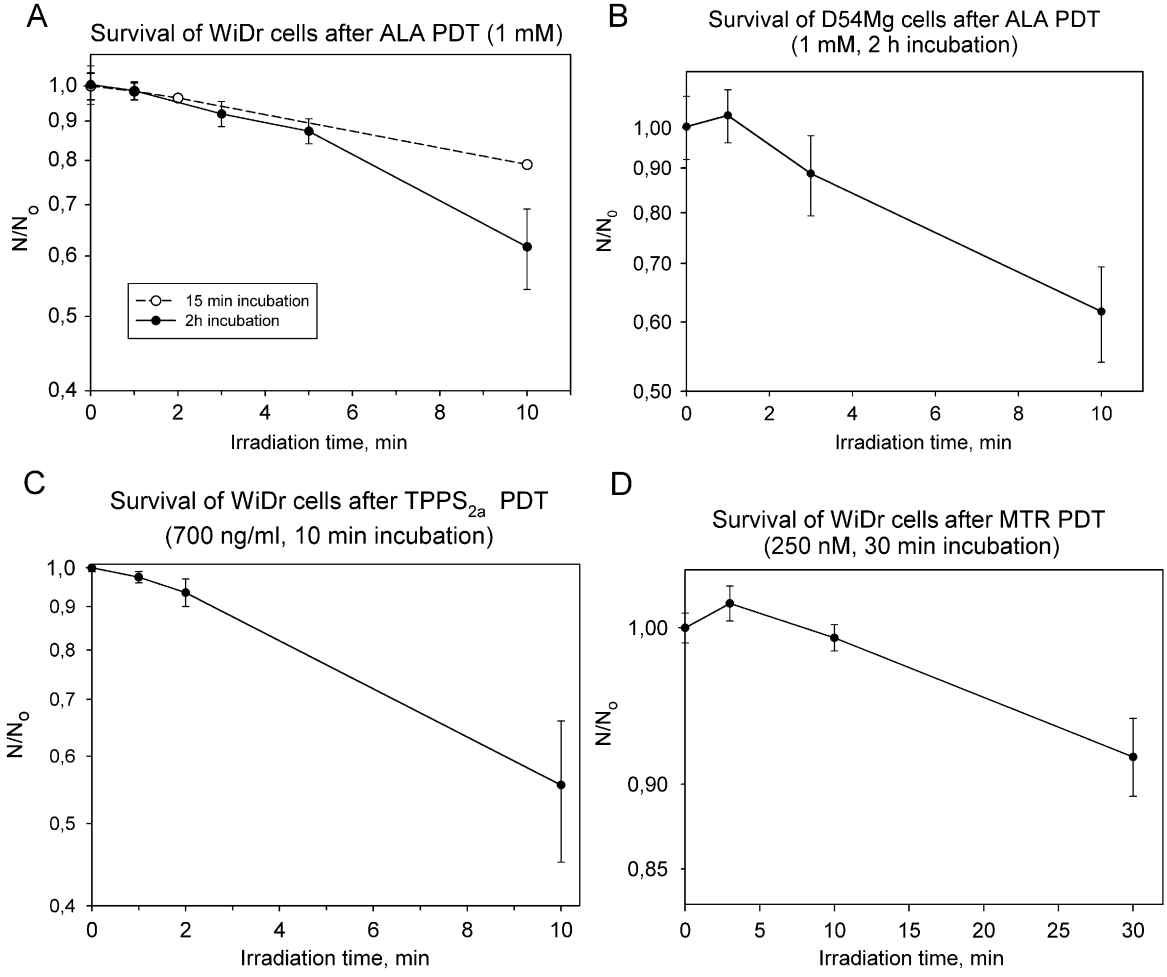

Fig. 1. PDT effect on cell survival. (A) Survival of WiDr cells after 15-min (open circles) or 2-h (closed circles) incubation with 1 mM ALA. (B) Survival of D54Mg glioma cells after 2-h incubation with 1 mM ALA. (C) Survival of WiDr cells after 10-min incubation with 0.9 μM $TPPS_{2a}$. (D) Survival of WiDr cells after 30-min incubation with 250 nM MTR. The values represent the mean ratio of the number of surviving photosensitized cells (N) to the number of untreated cells (N_0): $N/N_0 \pm$ S.E. Standard errors are not shown in cases when they are smaller than the symbol size.

FCS, 100 units/ml penicillin, 100 mg/ml streptomycin, and nonessential amino acids (Bio Whitacker). These cell cultures were incubated at 37 °C in a humidified 5% CO_2 atmosphere and subcultured twice a week using 0.01% trypsin in 0.02% EDTA. For the microscopic study approximately 10^4 cells were seeded into Falcon dishes (35 × 10 mm), in which they were incubated for 2–4 days before the staining procedure.

2.3. Light exposure

Cells incubated with ALA in tissue culture flasks or in cell cultivation wells were exposed to light from a bank of fluorescent tubes (Model 3026, Applied Photophysics, London, UK) with a fluence rate of 0.6 mW/cm^2. The emission of this lamp was mainly in the 370–450-nm region, with a peak at 405 nm, which is close to the

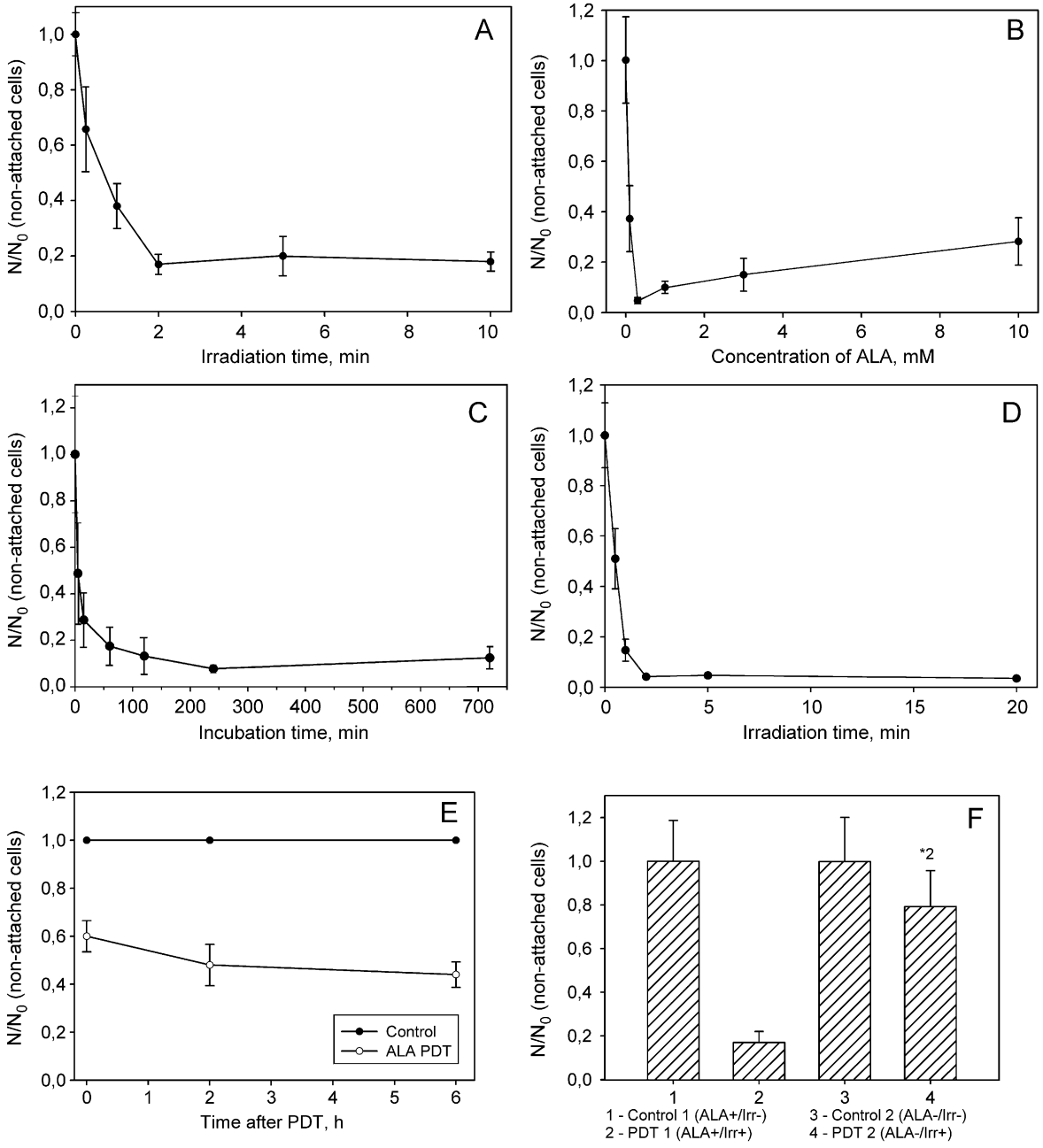

Fig. 2. Detachment of WiDr cells from the plastic substratum after ALA-PDT. The ratio of the number of detached photosensitized cells (N) to the number of detached cells in the control untreated sample (N_0) is plotted versus: light exposure after 2-h or 15-min incubation of cells with 1 mM ALA (A and D, respectively); ALA concentration (B; incubation time 2 h, light exposure 1 min); incubation time (C; ALA concentration 1 mM, light exposure 1 min); time after ALA-PDT (E; 1 mM ALA, 2 h incubation, 1 min irradiation). (F) The role of the effect of extracellular PpIX excreted by cells in ALA-PDT on cell detachment: replacement of incubation medium by fresh medium decreased efficiency of trypsinization (compare bars 4 and 2). The values represent mean $N/N_0 \pm$ S.E.

maximum of the Soret band of PpIX. Cells incubated with $TPPS_{2a}$ were irradiated with the LumiSource lamp (a bank of four light tubes, 4×18 W Osram L 18/67) emitting mainly the blue light (400–500 nm) with two close peaks at 436 and 450 nm and a fluence rate of 7 mW/cm^2. Cells stained by MTR were irradiated by a 150-W halogen lamp equipped with a combination of filters cutting off the green-blue (<560 nm) and infrared (>680 nm) parts of the lamp spectrum. The emission maximum was near 596 nm. The fluence rate was about 70 mW/cm^2 as measured by a calibrated photodiode.

2.4. Cell survival assay

In order to study the phototoxicity of the photosensitizers, 8×10^4 cells were inoculated in the plastic 12-well plates (Nunc, Roskilde, Denmark) containing 2 ml of medium RPMI 1640. After 3–4 days, cells were incubated with photosensitizers for a given time interval in the dark at 37 °C. Then in the dark room the dye-containing medium was replaced by the fresh dye-free medium and the cells were irradiated. After irradiation, the cells were incubated for 24 h at 37 °C. Then they were rinsed in 0.9% NaCl, fixed 20 min with 96% ethanol, and stained 20 min with methylene blue. The absorbance of the stained cells was then measured at 630 nm (methylene blue absorption band) using a Perkin-Elmer LS-50 B spectrofluorimeter equipped with a well plate reader accessory and a mirror under a well plate. It worked in the reflectance detection regime so that light was recorded after passing twice through the cell layer. Three to four wells were used for each light exposure. Relative survival fraction was calculated from:

$$N/N_0 = (\log I_b - \log I)/(\log I_b - \log I_0)$$

where I, I_0 and I_b—light intensities passed through experimental, control (unirradiated) and blank wells, respectively. This value was expressed as mean ± S.E.

2.5. Cell detachment assay

In the cell detachment experiment, 8×10^4 cells were subcultured in plastic 12-well plates (Nunc) containing 2 ml of medium RPMI 1640. In the next 3–4 days when the cell layers were almost confluent, photosensitizers were added and the samples were irradiated. Then the medium in the wells was replaced by 0.3 ml of trypsin/EDTA solution. The wells were incubated at 37 °C for 5–8 min until detachment of almost all cells in the untreated control samples. Then 1 ml of FCS-containing RPMI 1640 medium was added in each well to stop the proteolytic action of trypsin and 0.3-ml samples were taken. The number of cells detached from each well was counted by means of a hemacytometer. The effect of the trypsin treatment was estimated as the ratio N/N_0, where N and N_0—numbers of the detached floating cells in the experimental and control samples, respectively.

2.6. Fluorescence microscopy

Cells grown for 3–4 days in the 35×10-mm Falcon dishes were incubated with a photosensitizer dissolved in FCS-containing RPMI 1640 medium. Then the attached cells were washed three to five times with PBS, and studied with a Zeiss Axioplan microscope (Karl Zeiss, FRG) equipped with epifluorescence and a water immersion objective $40 \times$. In order to study the suspended cells, a droplet of the cell suspension was placed on a Falcon dish and covered with a coverslip. These cells were studied with an oil immersion objective $63 \times$. An HBO/100 W mercury lamp was used for fluorescence excitation. The 395–440-nm excitation filter and the dichroic beam splitter FT 460 were used for observation of PpIX and $TPPS_{2a}$ fluorescence. MTR fluorescence was observed using the 450–490-nm excitation filter and the dichroic beam splitter FT 510. The >630-nm long pass filter was used in all cases. Fluorescent images were taken by the cooled charge-coupled device (CCD) camera Photometrics (Princeton Instruments, Princeton, USA).

2.7. Intra- and extracellular porphyrin measurements

WiDr cells in the exponential growth phase were cultivated in 25-cm^2 flasks (5×10^5 cells/flask). After 72 h, these cells were washed with PBS and incubated for 2 h with 1 mM ALA in RPMI 1640 medium with or without FCS at 37 °C. For measurements of the intracellular PpIX content, the cells were washed with ice-cold PBS and resuspended in 1 ml of fresh PBS. The concentration of PpIX in cells was measured by means of a luminescence spectrometer (Perkin-Elmer LS50B, Norwalk, CT). The 407-nm fluorescence excitation wavelength corresponded to the absorption maximum of

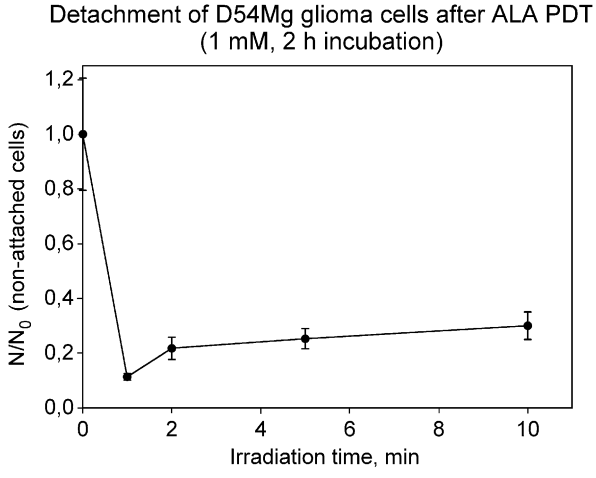

Fig. 3. Detachment of D54Mg cells from the plastic substratum after ALA-PDT. The ratio of the number of detached photosensitized cells (N) to the number of detached cells in the control untreated sample is shown (N_0). Trypsinization efficiency is plotted versus light exposure. ALA concentration 1 mM, incubation time 2 h. Values represent mean N/N_0 ± S.E.

PpIX. A long-pass filter (515 nm) was used to block the scattered excitation light. The fluorescence emission was measured at a wavelength of 636 nm corresponding to the maximum of PpIX fluorescence in this medium. For the measurements of the extracellular porphyrin content, 1 ml of culture medium was removed at a different time of the cell incubation period and the fluorescence was measured as described above. Variations in the cell density were less than 10%. This was checked by measurements of the scattered light at 600 nm. The fluorescence spectra were normalized to the cell number in each individual measurement and were corrected for the autofluorescence of cells not exposed to ALA. All measurements were performed in triplicate.

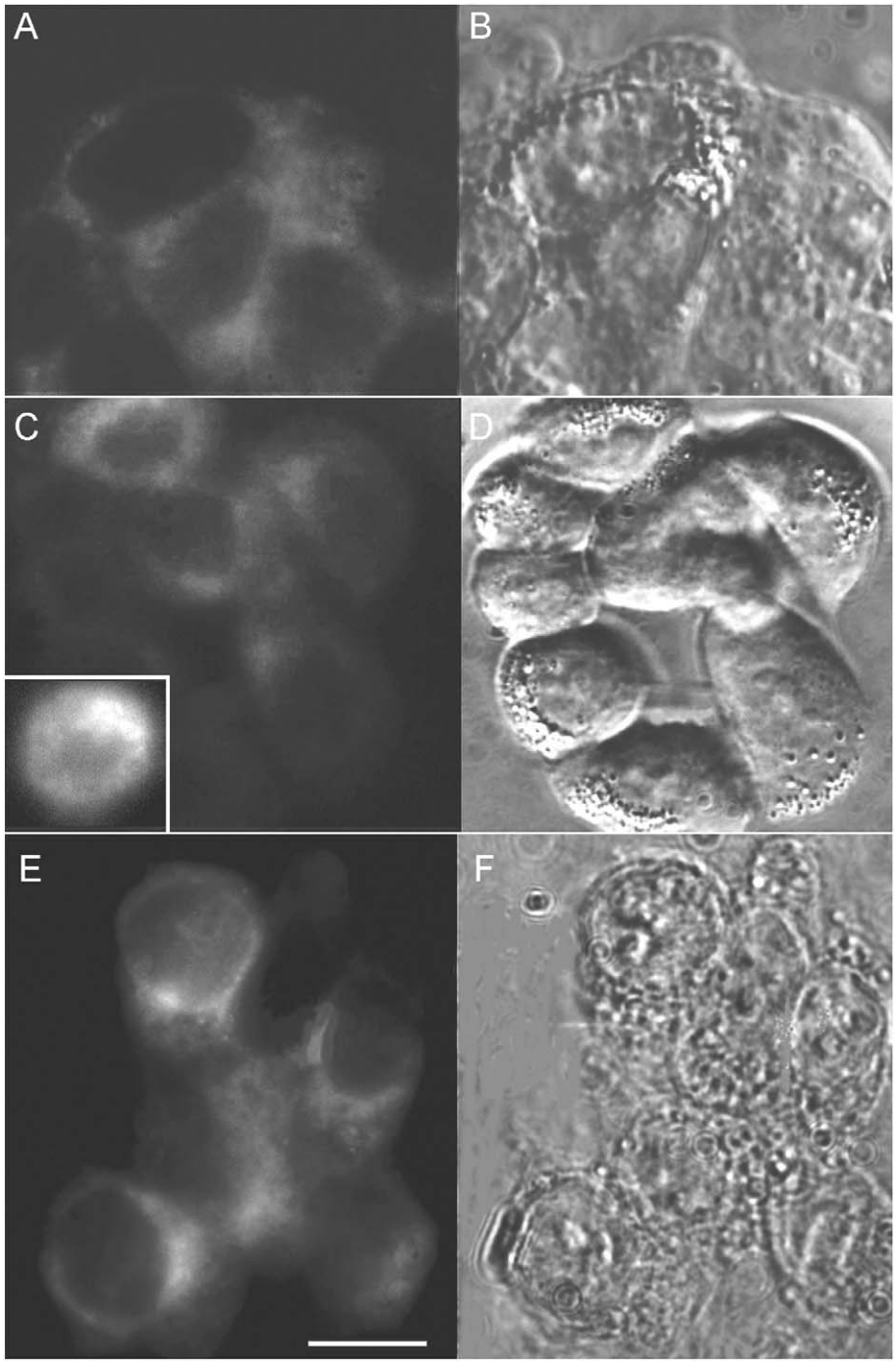

Fig. 4. Fluorescent micrographs of attached WiDr cells incubated with 1 mM ALA during 1 h (A), 2 h (B), or 28 h (C) in FCS-containing medium. The corresponding phase-contrast images are shown in panels (B), (D), and (F). The insertion shows the fluorescence of suspended WiDr cell incubated 2 h with 1 mM ALA in serum-free medium. The scale bar at (E) is 20 μm. Objective 63 ×, oil immersion.

2.8. Statistics

Standard statistical methods based on the Student's criterion were used. Results are expressed as mean ± S.E.

3. Results

3.1. Phototoxicity of ALA-induced PPIX, $TPPS_{2A}$ and MTR

Survival curves for WiDr cells photosensitized with ALA, $TPPS_{2a}$, or MTR are shown in Fig. 1. Light exposures of 4.7 or 3.7 min corresponded to $LD_{10} = 0.17$ or 0.13 J/cm^2 for WiDr cells incubated with 1 mM ALA for 15 min or 2 h, respectively (Fig. 1A). D54Mg cells were somewhat more sensitive to ALA-PDT (1 mM, 2 h incubation): a light exposure of 2.8 min corresponded to $LD_{10} = 0.1$ J/cm^2 (Fig. 1B).

LD_{10} for $TPPS_{2a}$-PDT (0.9 μM, 10 min incubation) and MTR-PDT (250 nM, 30 min incubation) of WiDr cells were 0.09 and 1.3 J/cm^2, respectively (2.6- and 35-min light exposures, Fig. 1C and D).

3.2. Trypsin-induced detachment of WiDr and D54Mg cells after ALA-PDT

ALA-PDT increased the resistance of WiDr and D54Mg cells to trypsin treatment. Only a minor fraction of the photosensitized cells detached from the substratum after such a treatment that, in the control samples, caused the detachment of almost all cells (Figs. 2 and 3). This effect was dose-, concentration- and incubation time-dependent (Fig. 2). It became significant at 1 min and was maximal after 2-min irradiation of WiDr cells incubated for 2 h with 0.3 mM ALA (Fig. 2A). Such light exposures were practically nontoxic with respect to cell survival (Fig. 1). Longer irradiation had no additional effect on cell detachment (Fig. 2A). The optimal ALA concentration was about 0.3–1 mM (Fig. 2B). Short-time incubation (15 min) of WiDr cells with 1 mM ALA also decreased the number of non-attached cells significantly (Fig. 2C and D). The effect was maximal at 4-h incubation; however, the results of 1–2-h incubation were not significantly different (Fig. 2C). Photoinduced changes in adhesion of WiDr cells to a substratum leading to increase in their resistance to trypsinization were stable and remained for at least 6 h after ALA-PDT (Fig. 2E).

3.3. Intracellular localization of PpIX produced from ALA

In order to elucidate the possible cellular targets for ALA-PDT, we studied the localization of ALA-produced PpIX in WiDr cells. After 1-h incubation of these cells with 1 mM ALA, PpIX fluorescence was localized in the cytoplasm, showing both the granular and diffuse distribution patterns (Fig. 4A). Unlike punctuate mitochondrial distribution [17],

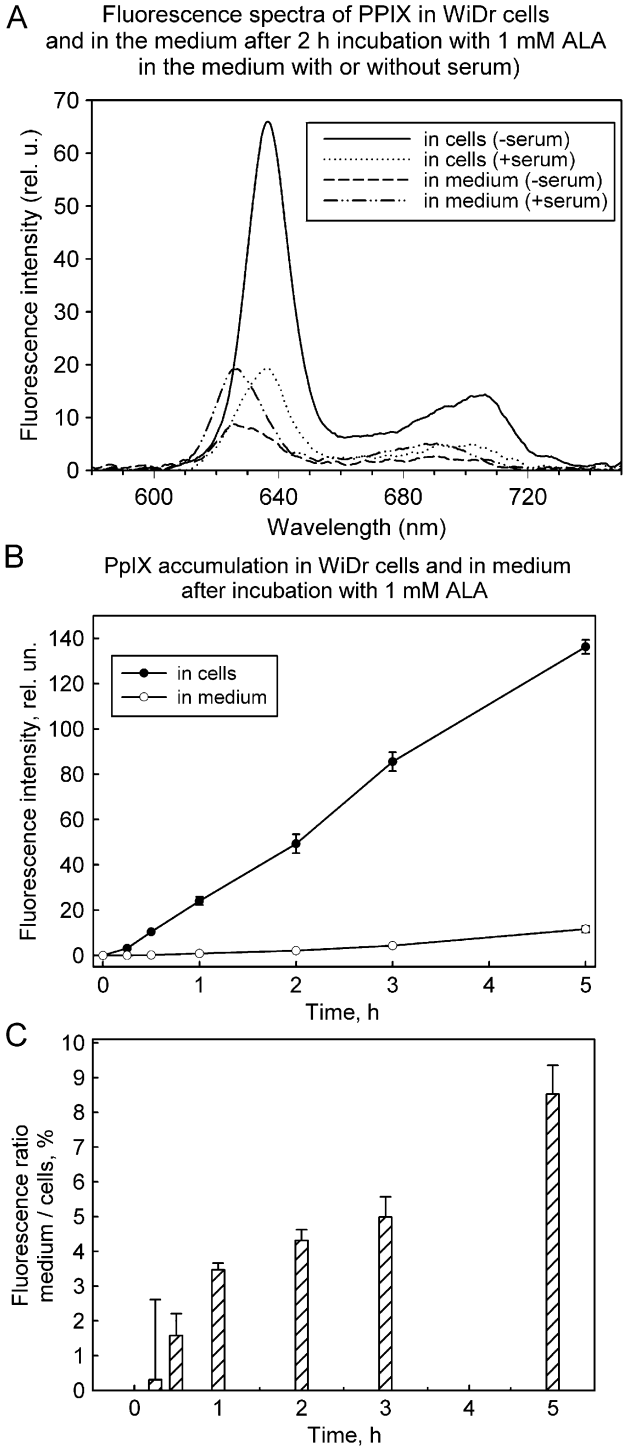

Fig. 5. Accumulation of PpIX in WiDr cells and in medium after 2-h incubation with 1 mM ALA in FCS-containing (+serum) or FCS-free (−serum) medium. (A) Porphyrin fluorescence spectra (excitation wavelength 407 nm). (B) Dynamics of PpIX accumulation in cells (closed circles) and in FCS-free medium (open circles). (C) Ratios of PpIX fluorescence in cells to fluorescence in the FCS-free medium versus incubation time. Values represent mean ± S.E. Standard error is not shown in the case when it is less than the symbol size.

such a pattern indicates a release of PpIX from mitochondria, where it is synthesized to a nonspecific cytoplasmic localization. No PpIX fluorescence was observed in the plasma membrane. After 2- and 28-h incubation, PpIX fluorescence was diffusely distributed in the cytoplasm, with an area of brighter fluorescence on one side of the perinuclear cytoplasm (Fig. 4C and E) where membranous organelles, such as endoplasmic reticulum, Golgi, and mitochondria, are mainly localized [17]. The plasma membrane did not fluoresce in these cells. It was difficult, however, to observe the plasma membrane in flattened cells, because its thickness was below the resolution limit of the optical microscope.

In order to reveal any fluorescence of PpIX in the plasma membrane, we studied suspended WiDr cells incubated for 2 h with 1 mM ALA. We assumed that the vertical parts of the plasma membrane in peripheral cell regions would be better seen in round suspended cells than in flattened cells. Unlike the detachment experiments, these cells were incubated in the FCS-free medium in order to avoid PpIX excretion to the culture medium and increase its fluorescence [18–22]. In the preliminary experiment, we found that the intracellular PpIX fluorescence was higher in the cells incubated in the FCS-free medium than in the presence of serum. The extracellular PpIX was, oppositely, lower in the FCS-free medium (Fig. 5A). However, no PpIX fluorescence was observed in the plasma membrane after 2-h incubation (Fig. 4C, insertion).

Direct measurements of PpIX fluorescence in WiDr cells and in the surrounding FCS-free medium during incubation with 1 mM ALA showed an increase of the extracellular PpIX concentration with time (Fig. 5B and C). Hence, a certain fraction of PpIX, which might not be seen by an optical microscope, could be retained in the plasma membrane during its efflux. This PpIX amount might be sufficient for photodamage of the plasma membrane and impairment of the adhesion processes. This assumption is supported by weaker PDT effect on the resistance of WiDr cells to trypsinization when the medium, in which cells were incubated with ALA, was replaced by fresh medium before irradiation (Fig. 2F).

PpIX localization in D54Mg glioma cells after 15-min incubation with 1 mM ALA was diffuse (Fig. 6A). However, some PpIX fluorescence was observed in the plasma membrane after 2-h incubation (Fig. 6C). This membrane fraction of PpIX might be responsible for the effect of ALA-PDT on the trypsinization efficiency in these cells (Fig. 3).

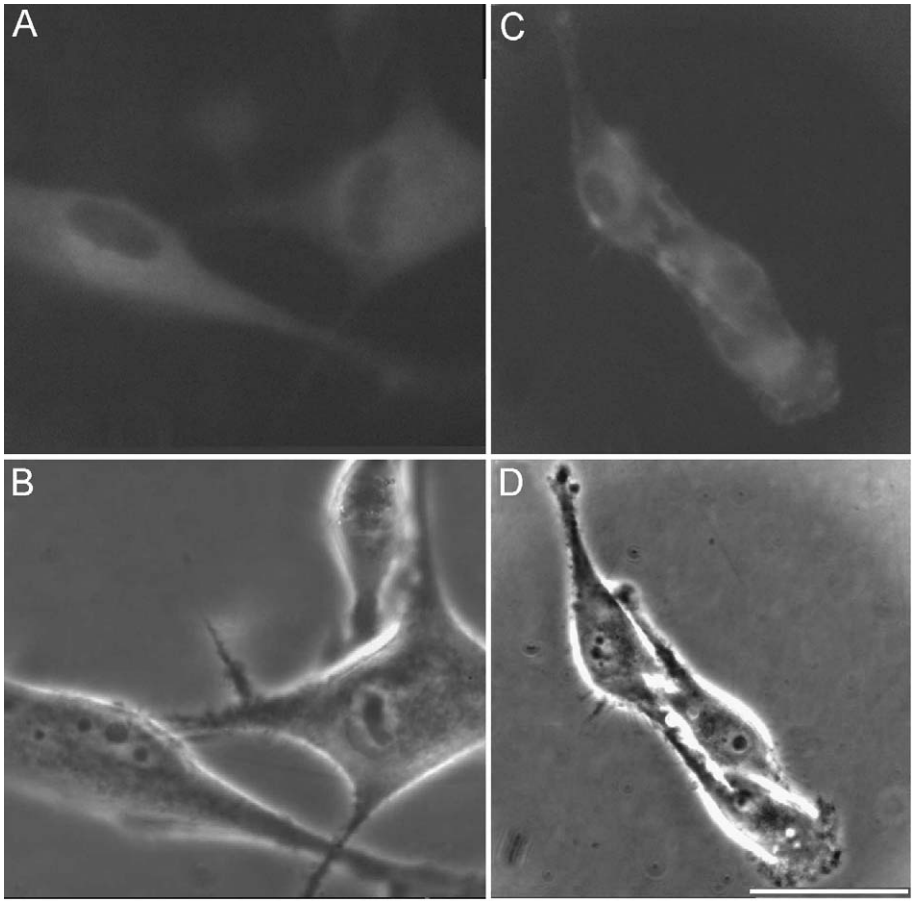

Fig. 6. Fluorescent micrographs of attached D54Mg cells incubated with 1 mM ALA during 15 min (A) or 2 h (C). Corresponding phase-contrast images are shown in panels (B) and (D). The scale bar in D is 20 μm. Objective 63 ×, oil immersion.

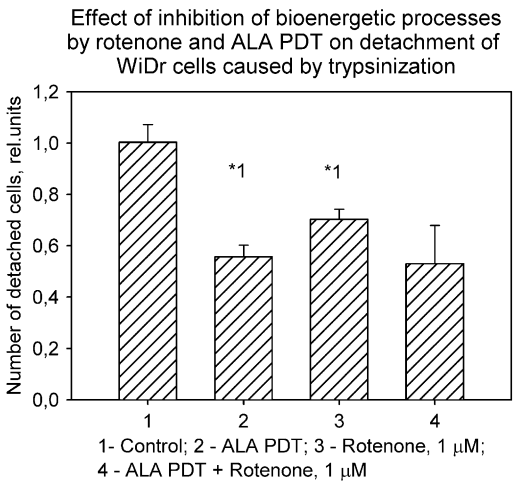

Fig. 7. Effect of inhibition of oxidative phosphorylation by 1 μM rotenone and ALA PDT on trypsin-induced detachment of WiDr cells from the substratum. Values represent mean $N/N_0 \pm$ S.E.*)—$P < 0.05$.

3.4. Effect of rotenone on ALA-PDT-induced inhibition of trypsin detachment

In order to study a possible involvement of mitochondrial bioenergetic processes in ALA-PDT-induced decrease of trypsinization efficiency, we used rotenone, a known inhibitor of oxidative phosphorylation. In our experiments, 1 μM rotenone alone reduced the number of cells detached after trypsinization like ALA-PDT (Fig. 7). However, rotenone did not modify the ALA-PDT effect on cell detachment (Fig. 7).

3.5. Trypsin-induced detachment of WiDr cells after $TPPS_{2A}$-PDT

The hypothesis that photosensitization of the plasma membrane may affect the adhesion processes, in particular enzymatic detachment, was supported by the experiment with short-time cell photosensitization with $TPPS_{2a}$. After

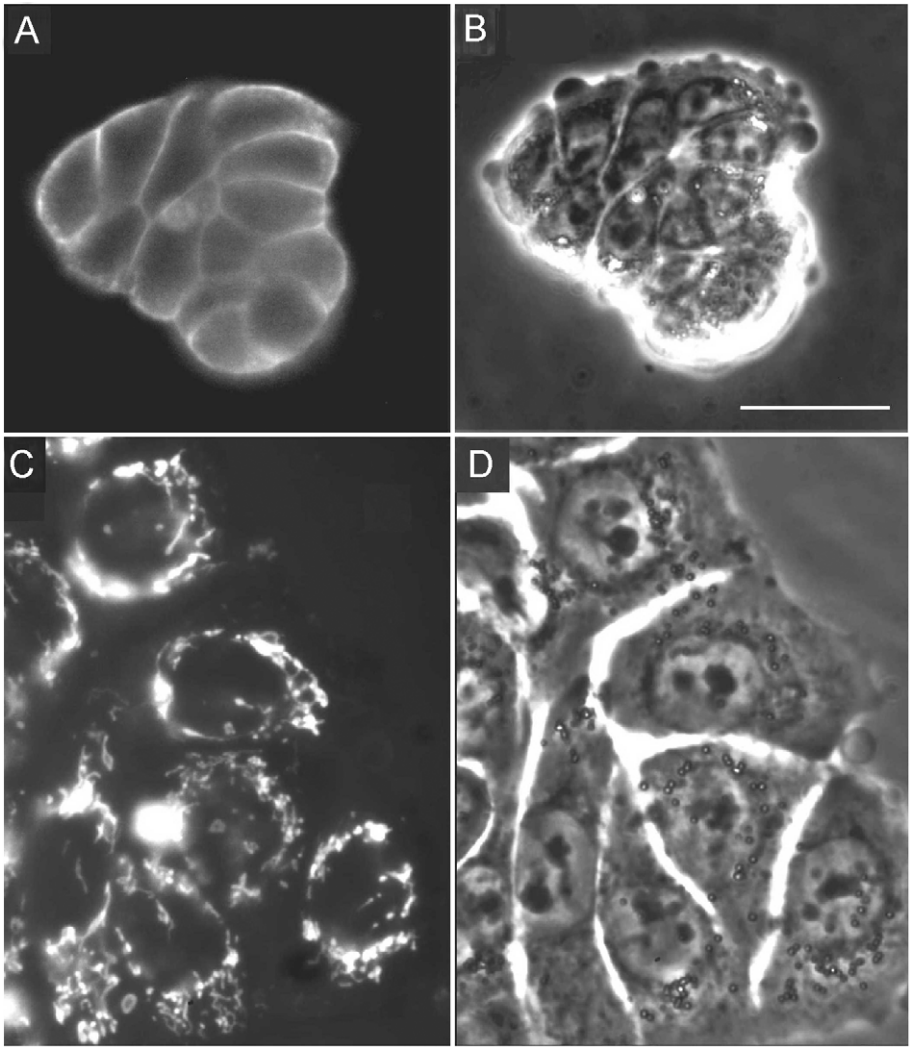

Fig. 8. Fluorescent micrographs of attached WiDr cells incubated with 0.9 μM $TPPS_{2a}$ during 10 min (A) or with 200 nM MTR during 20 min (C). The corresponding phase-contrast images are shown in panels (B) and (D). The scale bar in B is 20 μm. Objective 63 ×, oil immersion.

10-min incubation, $TPPS_{2a}$ (0.9 μM) penetrated poorly into WiDr cells and stained only the plasma membrane and perhaps a thin sub-membrane rim (Fig. 8A) containing $TPPS_{2a}$-loaded pinocytosis vesicles [23]. Under these conditions, photosensitization inhibited trypsin-induced cell detachment (Fig. 9A) like ALA-PDT. Therefore, direct PDT effect on the plasma membrane with nontoxic light exposures (10–40 s) influenced the adhesion processes.

3.6. Trypsin-induced detachment of WiDr cells after MTR-PDT

Can intracellular organelles such as mitochondria influence the adhesive processes occurring at the cell surface? In order to study this problem, we used MitoTracker Red, a mitochondria-selective fluorescent probe that can destroy mitochondria upon light exposure [24]. In WiDr cells MTR selectively stained mitochondria at a concentration of 200 nM and an incubation time of 15–20 min (Fig. 8C). Irradiation of WiDr cells stained for 20 min with 200 nM MTR inhibited trypsin-induced cell detachment (Fig. 9B) in the same manner as ALA-PDT or $TPPS_{2a}$-PDT. The applied light exposures (30–540 s) were not phototoxic (Fig. 1D). Therefore, not only deterioration of the plasma membrane but also damage of the intracellular organelles such as mitochondria may impair adhesive processes.

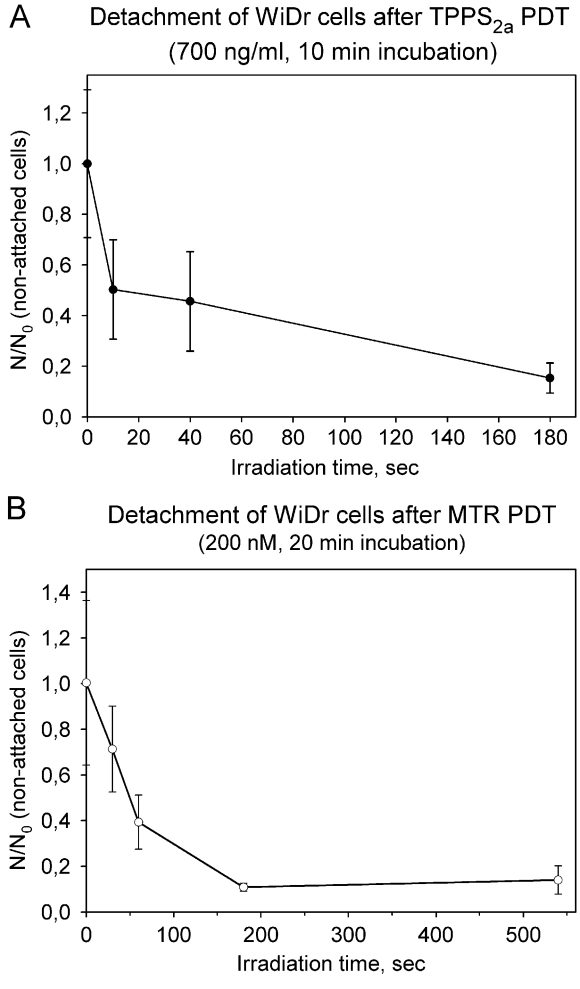

Fig. 9. Detachment of PDT-treated WiDr cells from the plastic substratum. The ratio of the number of detached photosensitized cells (N) to the number of detached cells in the control untreated sample (N_0) is plotted versus light exposure. (A) Photosensitization of cells with 0.9 μM $TPPS_{2a}$ (incubation time 10 min). (B) Photosensitization of cells with 200 nM MTR (incubation time 20 min). Values represent mean $N/N_0 \pm$ S.E.

4. Discussion

Adhesion processes occur at the cellular surface [25]. Trypsin can cleave the bonds between the cell surface proteins, integrins, and the substratum. This leads to cell detachment. In our experiments ALA, $TPPS_{2a}$, or MTR-mediated photosensitization inhibited trypsin-induced cell detachment from a plastic substratum. This result is in agreement with what has been found for PDT with hematoporphyrin [5,9,10] and zinc phthalocyanine derivatives [10]. Trypsinization is an obligatory procedure for bringing attached cells into suspension and is in common use in cell culture experiments. Therefore, a method to modify this procedure may be of general interest in cell biology. Furthermore, the present documentation of the photomodification of cell adhesion is of importance for our understanding of the basic mechanisms of photodynamic cancer treatment. Formation of cancer metastases includes detachment of malignant cells from the main tumour, penetration into blood vessels, travelling with blood flow and attachment to remote tissues where metastases are established. Both cell detachment and attachment are of importance for this process [11,12]. PDT not only destroys tumour tissues [1–4] but also reduces cancer metastases [13,14]. This is one of the advantages of this cancer treatment method. One of the reasons for this phenomenon may be inhibition of detachment of cancerous cells from the primary tumour.

The photoinduced increase in cell resistance to trypsinization occurred even for sublethal light exposures. We observed a significant inhibition of trypsin-induced detachment of WiDr (Fig. 2) or D54Mg (Fig. 3) cells after 1–2-min ALA-PDT. Such a treatment kills less than 2% of cells (Fig. 1). The same effect was observed when WiDr cells were photosensitized with $TPPS_{2a}$ or MTR (Figs. 1 and 9). The photoinduced modification of cell adhesion was stable: cells remained resistant to trypsinization during at least at 6 h after ALA-PDT (Fig. 2E).

PDT inhibition of trypsin detachment might be caused by direct photomodification of the cell-substratum bonds. Christensen et al. [5] assumed that PDT with hematoporphyrin derivative enhanced adhesion due to photoinduced cross-links between cellular proteins and proteins coating the substratum. Ball et al. [10] showed that PDT-induced decrease in trypsinization efficiency might be related to activation of tissue transglutaminase that cross-links proteins. Denstman et al. [9] guessed that photosensitization

makes trypsin cleavage sites near lysine and arginine inaccessible for the enzyme presumably due to photoinduced changes in the cell shape.

In order to elucidate the mechanisms of PDT-induced cell resistance to trypsinization, one should consider the cellular targets that are sensitive to PDT. Singlet oxygen, the main cytotoxic agent in photodynamic cell injury, diffuses no more than 10–20 nm during its lifetime [26]. Therefore, it acts in the vicinity of the photoexcited pigment molecule [26]. PDT-induced decrease in trypsinization efficiency was observed when the plasma membrane was selectively photosensitized by $TPPS_{2a}$ (Figs. 8 and 9). This is not unexpected since cell adhesion proteins such as integrins and cadherins are embedded in the plasma membrane [25]. The photodamage of the plasma membrane [27,28] could directly deteriorate these proteins. SH-groups of the proteins are very sensitive to PDT. These can be destroyed by sublethal light exposures [29].

Unlike the selective photosensitization of the plasma membrane with $TPPS_{2a}$, it is not clear, which cellular targets are altered by ALA-PDT. The endogenous photosensitizer PpIX is synthesized from ALA in mitochondria [3]. After synthesis it is released from these organelles, spreads throughout the cell and even goes out into the medium [18–21,30,31]. PpIX fluorescence has been observed in the cell membrane after an incubation longer than 4–5 h [18–20,30]. Probably, 2-h incubation, which was used in the most of our experiments, was too short for substantial photosensitization of the plasma membrane. As Fig. 4 shows, PpIX stained the cytoplasm but not the plasma membrane in WiDr cells after 1–2-h incubation with 1 mM ALA. However, Iinuma et al. [21] reported the presence of PpIX in the plasma membrane after 1-h incubation. The resolution of the fluorescence micrographs does not provide any good visualisation of the plasma membrane. We demonstrated that the PpIX efflux from WiDr cells into the medium became significant after 1 h or longer incubation with 1 mM ALA (Fig. 5). Therefore, a minor fraction of PpIX might be retained in the plasma membrane during its efflux. This is likely since PpIX is a lipophilic molecule. The presence of a small amount of the photosensitizer, not visualized by the fluorescence microscope, may be sufficient for direct photodynamic impairment of adhesion processes at the cellular surface. Unlike WiDr cells, we observed PpIX fluorescence in the plasma membrane of D54Mg cells after 2-h incubation (Fig. 4). Therefore, in these cells, ALA-PDT effect on the detachment from the substratum might be related to a direct damage of cell adhesion molecules.

At 15-min incubation of WiDr cells with 1 mM ALA, no PpIX was found in the medium, though PDT also inhibited trypsin-induced cell detachment in this case (Fig. 2B). The newly produced PpIX should still be in the cytoplasm, mainly in mitochondria, but not in the plasma membrane. Therefore, mitochondrial PpIX may be responsible for the PDT effect on cell detachment. The experiment with MTR-PDT (Figs. 8 and 9) clearly showed that not only photomodification of the cell surface but also selective photosensitization of mitochondria may inhibit the trypsin-induced cell detachment. Rotenone, an inhibitor of oxidative phosphorylation, decreased the efficiency of trypsinization like PDT did. Therefore, both treatment modalities might act on the same cellular targets. Mitochondria are not directly associated with the adhesion machinery. Hence, the photodynamic effect of MTR on cell detachment is an indirect effect. It is possible that photodynamic targeting of several cellular structures, the plasma membrane, mitochondria, and possibly the endoplasmic reticulum, might be responsible for the effect of ALA-PDT on enzymatic cell detachment. Our data are in agreement with the view that trypsin-induced cell detachment is an active multi-step processes requiring energy, signal transduction and reorganization of the cytoskeleton [32,33]. One can suggest that a mobile messenger establishes communication between the photoimpaired mitochondria and the adhesion molecules located at the plasma membrane. Ca^{2+} ions may serve as potential messengers. In fact, Ca^{2+} ions are known to be released from damaged mitochondria and endoplasmic reticulum [34]. The cytosolic Ca^{2+} level is quickly increased in photosensitized cells [35,36]. Ca^{2+} ions influence several signalling processes regulating cell adhesion [37–39]. The molecular mechanisms of the PDT effect on cell adhesion need, however, to be further investigated.

In conclusion, our data show that photosensitization of cells of two cultured cell lines, human WiDr adenocarcinoma cells and D54Mg glioblastoma cells, inhibits trypsin-induced cell detachment from a plastic substratum. This effect may be associated with direct photodynamic impairment of adhesion molecules at the cellular surface as in the case of $TPPS_{2a}$-PDT that selectively targets the plasma membrane, or with indirect deterioration of cell adhesion after selective photodynamic impairment of mitochondria by MTR-PDT. In the case of ALA-PDT, the endogenous photosensitizer PpIX could photosensitize both the plasma membrane and mitochondria and induce both direct and indirect effects leading to inhibition of enzymatic cell detachment. Thus, photosensitization of both the plasma membrane and mitochondria impairs trypsin-induced cell detachment.

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