Original Article

Photooxidative Action in Cancer and Normal Cells Induced by the Use of Photofrin® in Photodynamic Therapy

(photodynamic therapy / reactive oxygen species / superoxide dismutase)

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Abstract. Photofrin-mediated PDT was applied to malignant (A549 and MCF-7) and normal (HUV-EC-C) cells. The cells were incubated for different lengths of time after PDT. The cell responses to the therapy were examined by changes in SOD activity, phototoxicity, and mode of the cell death. PDT induced dynamic changes in SOD activity. Initially, an increase in SOD activity was observed, and after 6 hours of culture it decreased to the control level. Results obtained from MTT and the comet assay indicate that PDT caused immediate cell death via apoptosis in the A549, MCF-7, and HUV-EC-C cell lines. Our studies confirm that SOD is involved in the response of both cancer and normal cells to PDT.

Introduction

Photodynamic therapy (PDT) is an emerging modality in the treatment of neoplastic and non-neoplastic diseases. This therapy involves the administration of a photosensitizer, most often a porphyrin, and subsequent irradiation with light at the proper dose and wavelength. This combined treatment leads to the generation of reactive oxygen species (ROS), such as singlet molecular oxygen, hydroxyl radicals, and/or superoxide anions (Nowis et al., 2005).

Increasing evidence indicates that tumour cells can respond to photodynamic damage by undergoing cell death via apoptosis or necrosis (Plaetzer et al., 2003). Oxidative stress causes damage to cellular macromolecules such as nucleic acids, proteins, and lipids. The antioxidant defence system requires an increase in antioxidant enzyme activity, not only to cope with production of the initial radicals, but also the more toxic products of spontaneous free-radical reactions (Girotti, 2001). One of the most important antioxidant enzymes is superoxide dismutase (SOD). There are three isoforms of SOD. MnSOD is located in mitochondria, CuZnSOD is mainly found in the cytosol, and an extracellular SOD (EC-SOD) is bound to the extracellular matrix. Reportedly, SOD can partially prevent the photodestruction caused by PDT (Golab et al., 2003; Dolgachev et al., 2005).

The mechanism of the photodynamic reaction in tissues has not been fully elucidated (Kim et al., 2001). The anti-cancer effects of PDT are thought to occur at two different levels: direct lethal effects on tumour cells and vascular impairment that limits blood supply to the region (Almeida et al., 2004). One of the suggested mechanisms of PDT destruction of tumours is damage to the vascular endothelium and tumour cell destruction as a result of structural damage to capillaries and functional disturbance in the microcirculation. The course of changes constantly observed in cells under the influence of PDT can be different, depending on oxygen accessibility, the type of photosensitizer and its concentration, the intensity of light, as well as the kind of cell. It has been demonstrated recently that PDT is most destructive to membranes, particularly of mitochondria and lysosomes (Lavi et al., 2002; Mak et al., 2004). The release of cytochrome c following mitochondrial photodamage could therefore represent a primary event in apoptotic cell death in which the caspase cascade is involved. The disintegration of cellular structures and the modulation of genetic information induced by PDT can direct cancer cells to a death pathway (Pogue et al., 2001; Rancan et al., 2005).

The aim of this study was to evaluate the Photofrin® (Ph)-mediated photodynamic effect on malignant (A549 and MCF-7) and normal (HUV-EC-C) cell lines. The SOD activity was investigated since it is involved in the response to oxidative stress. The efficacy of PDT was

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Abbreviations: MTT – 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl--SH-tetrazolium bromide, PDT – photodynamic treatment, Ph – Photofrin®, ROS – reactive oxygen species, SOD – superoxide dismutase.

estimated using the 3-(4,5-dimethyl-thiazoyl)-2,5-diphenyl-SH-tetrazolium bromide (MTT) test and the neutral comet assay.

Material and Methods

Cell lines

Two human malignant cell lines (A549, MCF-7) and a normal human umbilical vein endothelial cell line (HUV-EC-C) were used. The human lung carcinoma cell line A549 was initiated in 1972 by D. J. Giard et al. through an explanted culture of lung carcinomatous tissue from a 58-year-old Caucasian male (Giard et al., 1973). The human breast adenocarcinoma cell line MCF-7 is derived from a pleural effusion taken from a 69-year-old Caucasian female with metastatic breast cancer.

Cell culture

The malignant cell lines (A549, MCF-7) were grown in MEM medium with addition of 10% foetal bovine serum in 25-cm^2 Falcon flasks. The HUV-EC-C line was grown in Ham's F12K medium with 2 mM L-glutamine adjusted to contain 1.5 g/l sodium bicarbonate and supplemented with 0.1 mg/ml heparin and 0.03–0.05 mg/ml endothelial cell growth supplement (ECGS) and 10% foetal bovine serum. The cells were maintained in a humidified atmosphere at 37°C and 5% CO_2. For the experiments the cells were removed by trypsinization and then washed twice with PBS.

Photodynamic treatment

The cells were treated with 15 and 30 μg/ml Photofrin® (Ph; QLT PhotoTerapeutics, Inc., Vancouver, Canada) in complete media for 4 h in the dark. Then they were irradiated by a light dose of 6 J/cm^2 using a lamp (OPTEL Fibre Illuminator, Opole, Poland) with polarized light (fluence rate at the level of the cell monolayer: 10 mW/cm^2) and a red filter (λ_{max} = 632.8 nm). All irradiations were performed at room temperature. After irradiation the cells were incubated in a humidified atmosphere at 37°C and 5% CO_2 for 5 min, 3 h, or 6 h.

MTT assay

The viability was determined by MTT assay (Sigma, Saint Louis, MO; In Vitro Toxicology Assay). For the experiment the cells were seeded into 96-well microculture plates at 1 x 10^4 cells/well and allowed to attach overnight. The medium was removed and replaced with fresh medium with or without Ph. The cells were incubated for 4 h and then irradiated. After PDT the cells were washed twice in PBS and treated according to the manufacturer's protocol. The absorbance was determined using a multiwell scanning spectrophotometer at 570 nm (Labsystem Multiscan MS type 352, Helsinki, Finland). Mitochondrial function was expressed as a percentage of viable treated cells relative to untreated control cells (without light and Ph). Experiments were repeated four times.

Neutral Comet Assay

For the detection of DNA fragmentation associated with apoptosis, the neutral comet assay was used as described by Collins (2002). The cells, at a concentration of 1x10^5/ml, were mixed with low-temperature-melting agarose (Sigma) at a ratio of 1 : 10 (v/v) and spread on a slide. The slides were submerged in pre-cooled lysis solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base, and 1% Triton X-100) at 4ºC for 60 min. After lysis and rinsing, the slides were equilibrated in TBE solution (40 mM Tris/boric acid, 2 mM EDTA, pH 8.3), electrophoresed at 1.0 V/cm^2 for 20 min, and then silver staining was performed (Yasuhara et al., 2003). For scoring the comet pattern, 100–200 nuclei on each slide were counted and assigned to a category from 0 to 4, depending on the relative staining intensity of DNA in the tail (0 = no DNA in tail: 4 ≥ 75% of DNA in tail). Experiments were repeated three times.

Determination of SOD activity

After photodynamic treatment *in vitro*, the cells were removed by trypsinization and washed twice in PBS. Then the cells were suspended in 50 mM phosphate buffer, pH 7, with a mixture of protease inhibitors. The total intracellular SOD activity was measured using a Ransod kit purchased from Randox Laboratories Ltd. (Antrim, UK) according to the manufacturer's protocol. Experiments were repeated three times.

Statistical analysis

Significance of the differences between the mean values of the different groups of cells was assessed by the Student's *t*-test; values of $P < 0.05$ were taken to imply statistical significance.

Results

MTT assay

The function of the cellular mitochondria was measured 5 min, 3 h, and 6 h after PDT, with treatment only with Ph as a control. Both the A549 and MCF-7 cancer cells showed significant disorder in the mitochondria function within 5 min after PDT when 30 μg/ml Ph was used, whereas treatment with only Ph at the highest dose did not affect the viability of either cell line after 6 h (Fig. 1A, B). The MCF-7 cells were more sensitive to PDT after 6 h than A549 cells.

The normal HUV-EC-C cells also showed significant disorder in mitochondria function under the same PDT conditions as the cancer cells. The viability of the cells decreased to 64% 5 min after PDT treatment with 15 μg/ml of Ph; after 6 hours only 3% of the cells survived (Fig. 1C).

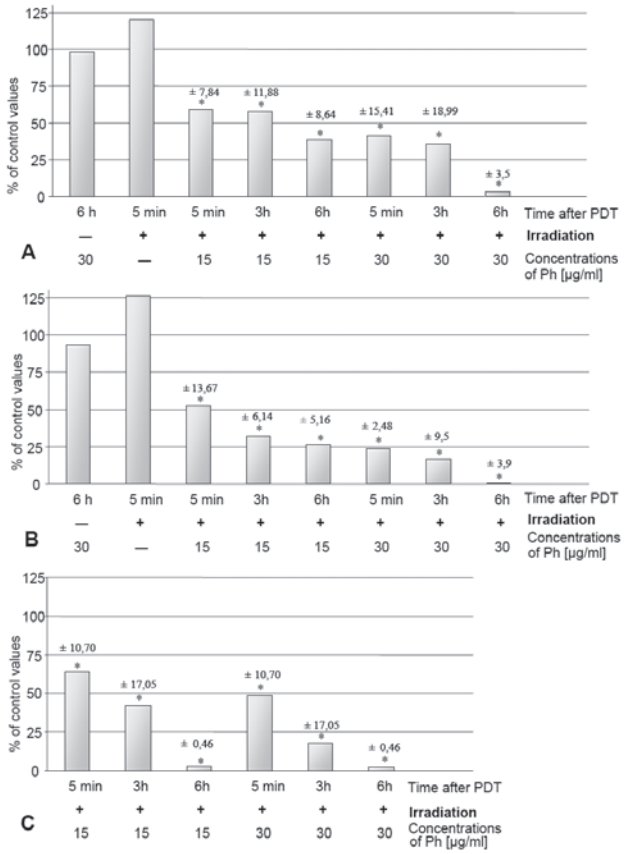

Fig 1. MTT assay after Ph-based photosensitization in (A) A549, (B) MCF-7, and (C) HUV-EC-C cells. The cells were incubated with 15 or 30 μg/ml of Ph for 4 h and then irradiated at 6 J/cm^2.
∗ P < 0.05 statistically significant compared with the control cells without PDT

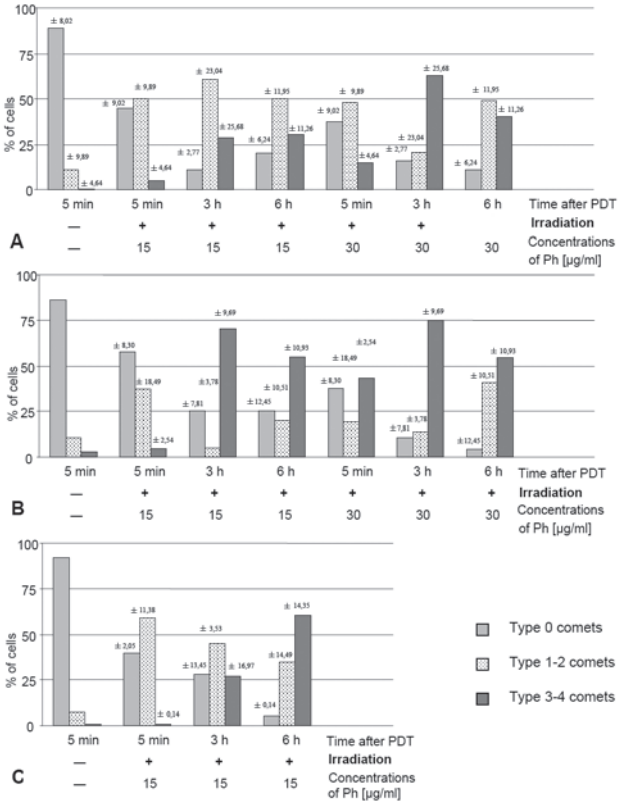

Fig. 2. Effect of Ph-based photosensitization on DNA degradation in (A) A549, (B) MCF-7, and (C) HUV-EC-C cells. The cells were incubated with 15 or 30 μg/ml of Ph for 4 h and then irradiated (6 J/cm^2).

Neutral comet assay

The influence of PDT on the mode of cell death was measured by DNA fragmentation. To describe this process, the neutral comet assay was performed. Our results are shown in Fig. 2 (A – A549, B – MCF-7, and C for HUV-EC-C). Under neutral conditions, breaks in single-stranded nuclear DNA are not observed as comets because each damaged strand is still associated with an undamaged one and is noted as a comet of type 0. Apoptotic cells are categorized as type 3 or 4. Comets of types 1 and 2 reflect undefined nuclei. Most apoptotic cells were detected after 3 h in cancer cells and after 6 h in normal cells (Fig. 2).

We observed many A549 cells with undefined nuclei. The apoptotic programme was probably triggered in these cells and DNA double-strand breaks occurred in them (Fig. 2A). The most apoptotic nuclei were noted after 3 h in MCF-7 cells and at the highest dose of PDT in the A549 cell line (Fig. 2A, B). After 6 h of incubation after PDT, a decrease in the number of apoptotic cells was observed (Fig. 2B, C).

A characteristic distribution of comets was noticed, with a relatively high number of comets of types 0 and 3–4 and a low amount of type 1–2, in MCF-7 cells treated with 15 μg/ml Ph (3 h after irradiation) and with 30 μg/ml Ph (5 min after irradiation) (Fig. 2B, C).

The normal cell line HUV-EC-C appeared sensitive to PDT. The number of apoptotic cells increased to 60.3% at 6 h after photodynamic treatment (Fig. 2A).

SOD activity

We observed that SOD activity significantly increased after 5 min of irradiation, remained elevated above the level of the controls at 3 hours, and then decreased after 6 hours (Fig. 3). The rise and fall were the most intense for the highest PDT dose, i.e. 30 μg/ml of Ph and 6 J/cm^2 irradiation. For the other conditions of PDT, the changes in SOD activity were less significant. The change in SOD activity in MCF-7 cells after PDT is more obvious than in A549 cells.

SOD activity was measured in the HUV-EC-C line treated with 30 μg/ml Ph and irradiated with 6 J/cm^2. The enzyme activity rose immediately after irradiation

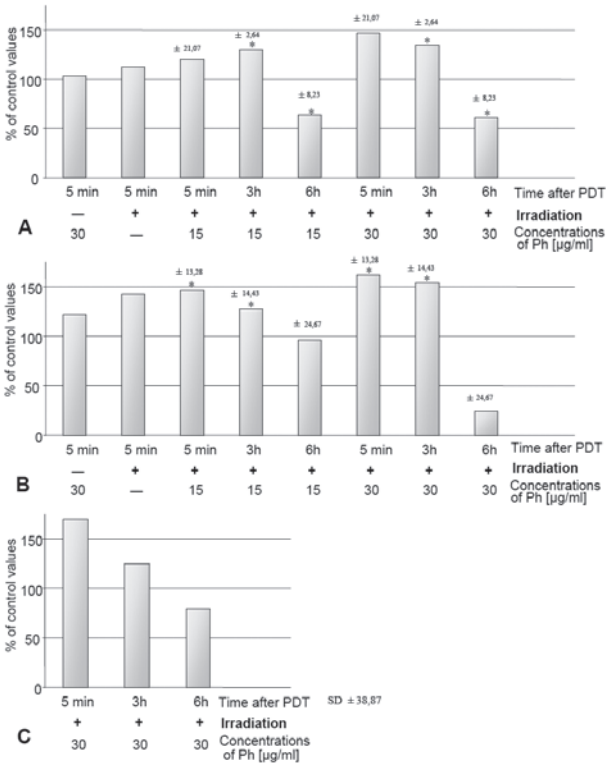

Fig. 3. SOD activity in (A) A549, (B) MCF-7, and (C) HUV-EC-C cells after Ph-based photosensitization. The cells were incubated with 15 or 30 μg/ml of Ph for 4 h and then irradiated (6 J/cm^2).
∗ $P < 0.05$ statistically significant compared with the control cells without PDT

to 170% of the control level and then gradually decreased after 6 h of incubation, reaching 80% of the level of the control cells without treatment.

Discussion

In the last decades the knowledge of the biochemical pathways induced during photodynamic therapy has advanced importantly. With the increasing number of *in vitro* and *in vivo* studies using various types of cells, photosensitizers and their concentrations, and times of treatment and light exposure, some rules of the photodynamic process can be defined. In the present study we used different approaches to examine the cell death induced by PDT using Ph as photosensitizer in human epithelial cancer cell lines A549 and MCF-7 and the normal endothelial cell line HUV-EC-C. Immediate cell death (within 5 min) was observed in all the studied cell lines at both concentrations of Ph (15 and 30 μg/ml). Prompt cell death (within 5 min) was also noted by Das et al. in two human cervical carcinoma cell lines treated with Ph-PDT (Das et al., 2000). Similarly, immediate cell death in human HaCaT keratinocytes after Ph-PDT was observed using the MTT assay by Woods et al. The reduction in mitochondrial function indicated that damage to these organelles occurred after PDT with the applied doses of Ph (Woods et al., 2004). This result was consistent with other reports that the inhibition of mitochondrial function could be connected with the release of cytochrome c and loss of $\Delta\Psi m$, which were the main causes inducing the apoptotic pathway (Granville et al., 1998; Kessel and Luo, 1998; 1999; Kessel et al., 2001; Lam et al., 2001; Brendler-Schwaab et al., 2004).

The results of the MTT test suggest that A549 cells are the least sensitive to Ph-PDT among the tested cell lines. Jiang et al. (2002) studied two human glioma cell lines, U87 and U25ln, treated with Ph-PDT. Ph cytotoxicity was determined using MTT. IC_{50} was exceeded at doses of 10 μg/ml of Ph in both lines, but at doses of 5 μg/ml of Photofrin® only in the U25ln cells. These results confirmed our observations that cell viability after PDT is dependent on the concentration of the photosensitizer. Cytotoxicity studies on PDT with Photofrin® and other porphyrins on a human colon adenocarcinoma cell line (HCT116) were performed using the MTT assay by Banfi et al. (2004). These results indicate that 3,4,5-trimethoxyphenyl, 3OH- and 4OH-phenyl, and sulfonamidophenyl derivatives are significantly more potent than Photofrin®. Therefore, cytotoxicity is also dependent on photosensitizer type.

In this study it was shown that the normal cell line HUV-EC-C is sensitive to PDT under the same conditions as malignant cell lines. This cell line is derived from endothelial cells which build blood vessels supporting solid tumours. In clinical treatment it offers the opportunity to destroy both cancer cells and the blood vessels that supply them.

The MTT test provides some information concerning the function of mitochondria; therefore it did not assess the late, irreversible changes that point to the mode of cell death. The comet assay under neutral conditions allows detection of the DNA double-strand breaks characteristic of apoptosis. Our study showed that DNA damage to MCF-7 cells appears very quickly in comparison with A549 and HUV-EC-C cells. The comet assay provides evidence of Ph-PDT-induced cell death via apoptosis in the studied malignant and normal cells. Woods et al. (2004) observed that Ph-PDT induced a dose-related increase in DNA migration with increasing irradiation dose in HaCaT cells. The breaks produced in the higher irradiation doses (10 and 25 J/cm^2) were probably caused in part by cell death.

There is much evidence demonstrating apoptosis and necrosis in PDT-treated cells (Ding et al., 2004; Kaneko et al., 2004; Marchal et al., 2005). Rapid apoptosis induction after the release of apoptosis-inducing factor and cytochrome c was also demonstrated (Granville et al., 2001; Kessel and Castelli, 2001). In our study, a similar rapid effect of PDT on apoptosis induction was observed in two cell lines. The use of HpD-PDT for a

short time of light exposure in the therapy in most cases induced apoptosis in normal cells (3T3 Balb), but there were both apoptotic and necrotic nuclei in malignant cells (A431). In any event, PDT factors provoked necrosis after higher doses of light (Marcinkowska et al., 2001). Thus, it seems that PDT may induce apoptotic or necrotic cell death, or the two types of cell death simultaneously.

In this study the comet assay showed that 6 h after PDT the number of type 3–4 comets, representing nuclei with DNA double-strand breaks, decreased in MCF-7 cells and, at the highest dose, in A549 cells. This event could be explained by the assumption that some cells could repair the DNA breaks. Other authors observed that murine glioblastoma cells were capable of significant DNA repair 4 h after m-THPC-PDT (Rousset et al., 2000). DNA damage and repair in Gorlin syndrome and normal fibroblasts after aminolaevulinic acid PDT have been noted (Haylett et al., 2003).

In our study the total intracellular SOD activity in the cells after PDT was examined. A rapid increase in SOD activity was observed immediately after exposure to the light. Then, six hours later, the activity fell in most cases below the level of controls. These results may suggest that after six hours the cells discontinue the struggle with oxidative stress and undergo death. However, the increase in SOD 5 min post PDT is difficult to explain. This time seems to be too short for the activation of transcription of genes encoding SOD. The event could be explained by the existence of an endogenous inhibitor of SOD. The inactivation of this inhibitor could be a clarification of this issue. Unfortunately, no one has yet confirmed the occurrence of such an inhibitor. A sudden increase in SOD activity was also observed by Johnson and Pardini (1998) in the EMT6 mouse mammary carcinoma cell line following exposure to light with hypericin. Cu,ZnSOD activities peaked within 0.5 h following PDT. MnSOD activity increased immediately following irradiation. Then, the level of MnSOD activity reached the control level and after that declined. Dolgachev et al. (2005) showed that MnSOD affects the sensitivity of cells to Pc 4-PDT-initiated apoptosis. The lack of MnSOD leads to potential ceramide accumulation and apoptotic sensitivity to PDT. MnSOD could be a potential molecular target for regulating cellular sensitivity to PDT, which could lead to therapeutic interventions (Haylett et al., 2003). Golab et al. (2003) showed that Ph II-based PDT induced expression of MnSOD, but not of Cu,ZnSOD, in murine colon-26 (C-26). The inhibition of SOD activity in tumour cells induced an increase in the cytotoxic effect of PDT. In contrast, transient transfection with the MnSOD gene, but not the Cu,ZnSOD gene, resulted in a decreased effectiveness of PDT (Rousset et al., 2000). These results suggest that MnSOD, but not Cu,ZnSOD, plays the leading role in the response to PDT and that mitochondria impairment may be a critical factor in phototoxicity. These SODs appear to be important antioxidative enzymes that regulate the sensitivity of cancer cells in some therapeutic methods such as PDT (Huang et al., 2000).

In our previous studies we examined PDT using a haematoporphyrin derivative (HpD) on 3T3 Balb and A431 cells. The factors of PDT (irradiation and concentration of the photosensitizer) caused dynamic changes in SOD and catalase (CT) activity, which were dependent on the intensity of these factors. These results strongly suggest that HpD influences generation of ROS, which is a signal for the development of morphological changes (apoptosis or necrosis) in normal and malignant cells (Marcinkowska et al., 2001).

Understanding the molecular events that contribute to PDT-induced apoptosis, and the way in which cancer cells can evade apoptotic death, should enable a more rational approach to drug design and therapy. Reaction pathways, cytotoxic effects, and cytoprotective mechanisms in cells after PDT are dependent on the conditions of this therapy.

The present study has presented evidence that these changes contribute to cell death by way of apoptosis. It seems that SODs are involved in the cell death and damage to mitochondria. There are few data considering the anti- and prooxidative response of the cells after PDT. There is a need to determine the role of antioxidant defence systems during PDT for improving the efficacy of this therapy.

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