

Aim of the study: Photodynamic therapy (PDT) is an approved, minimally invasive and highly selective therapeutic approach to a variety of tumors. It is based on specific photosensitizer accumulation in the tumor tissue, followed by irradiation with visible light. The photochemical interactions of the photosensitizer, light and molecular oxygen produce singlet oxygen and other reactive oxygen forms. The imbalance between ROS generation and antioxidant capacity of the body gives rise to oxidative stress in the cell, which initiates cell death in PDT. The aim of this study was to investigate the effect of photodynamic reactions in human melanoma cell lines.

Material and methods: Photofrin® (Ph) was used for the photodynamic reaction *in vitro* as a photosensitizer. The primary cell line was MEWO cell line (granular fibroblasts), derived from a human melanoma. As a recurrent cell line we used Me45 cell line, derived from a lymph node metastasis of skin melanoma. We compared cell viability (MTT assay) to determine the effectiveness of applied therapy. The intracellular distribution of photosensitizer (Photofrin) and localization of mitochondria (MitoTracker Green) were detected by confocal microscopy.

Results: We observed that Me45 and MEWO cell viability was dependent on the time of incubation after irradiation. In the recurrent cell line Ph accumulated mainly in the mitochondrial membranes and in MEWO cells also in the cytoplasm. The primary melanoma cell line exhibited significantly reduced cellular proliferation (below 50%) after photodynamic reaction with Ph.

Conclusions: The applied photodynamic reaction was more effective in primary melanoma cells. Additionally, mitochondrial localization of Ph can lead to disturbances of mitochondrial transmembrane potential and finally to release of apoptotic proteins.

Key words: photodynamic reaction, skin cancer, oxidative stress, Photofrin®.

Comparison of the influence of photodynamic reaction on the Me45 and MEWO cell lines *in vitro*

Anna Choromańska¹, Jolanta Saczko¹, Julita Kulbacka¹, Iwona Kamińska², Nina Skotucka¹, Michał Majkowski³

¹Department of Medical Biochemistry, Wrocław Medical University, Wrocław, Poland

²Department of Pathology and Clinical Cytology, Wrocław Medical University, Wrocław, Poland

³Laboratory of Cytochemistry, Biotechnology Faculty, University of Wrocław, Wrocław, Poland

Introduction

Malignant melanoma (lat. *melanoma malignum*) is a tumor derived from melanocytes – skin pigment cells responsible for melanin production [1, 2]. It is the most severe skin neoplasm as it may grow rapidly and metastasize through blood and lymphatic vessels [3–5]. Previous attempts of multi-agent chemotherapy for treatment of such malignant changes provided an objective response rate of 20% [6]. In addition, chemotherapeutic agents often lead to secondary tumor resistance [7]. Current melanoma treatment is based mainly on surgical removal by a large marginal of safety (5 mm to 2 cm). The most common chemotherapeutic agents used in melanoma treatment are melphalan and dacarbazine [6, 8]. There is no fully effective treatment and the application of photodynamic therapy (PDT) opens up new perspectives in the therapy of this type of cancer. Photodynamic therapy is based on cooperation of three factors: photosensitizer, which accumulates only in the tumor; light of the appropriate wavelength; and oxygen dissolved in the tissue. Photosensitizer is activated after exposure to appropriate light wavelength. The excitation energy is transferred from the absorption site and then for the production of molecular oxygen [8, 9]. The photochemical interactions of the photosensitizer, light and molecular oxygen produce singlet oxygen and other forms of reactive oxygen species (ROS). Photodynamic therapy induces disintegration of cellular structures and modulation of genetic information. These changes are caused by oxidative stress and cytotoxic effects in the cell. In recent studies of Kästle *et al.* the authors observed a high level of ROS after 5-ALA-PDT in WM451LU melanoma cell line [10]. It was presented that WM451LU cells are more susceptible to PDT than normal human keratinocyte cells. The authors suggest that it could be induced by altered metabolism of heme in cancer cells. Leibovici *et al.* have shown that in cancer cells activity of porphobilinogen deaminase increased significantly as compared to normal cells [11]. Furthermore, Dailley and Smith showed a significant decrease of ferrochelatase activity in various cancer cells [12]. These metabolic changes cause the accumulation of protoporphyrin photosensitizers in cancer cells. Therefore, the concentration of accumulated photosensitizer is significantly higher than in normal cells. Today, the “gold standard” is surgical removal of melanoma. However, there are cases, especially in the elderly, in which it is not possible to perform the operation. Moreover, some melanomas are completely inoperable [13]. Especially the surgical treatment of lentiginous melanoma in the elderly is often problematic [14]. For this reason PDT may be an alternative method of cancer treatment.

The aim of this research was to compare the influence of photodynamic action with Photofrin® on survival of melanoma cells derived from primary

and secondary cell lines *in vitro*. Photosensitizer distribution was also evaluated in both types of cells.

Material and methods

Cell lines

In the research a secondary melanoma line called Me45 was used. The cell line was obtained from the Oncology Centre Gliwice, where the line was derived from a 35-year-old woman's lymph node cells. The primary line used in experiments was MEWO cell line, purchased from CLS (Cell Lines Service, Germany). The cells were cultured in culture flasks (25 cm³, Falcon) in DMEM (Sigma) with 2 mmol/l glutamine and 10% fetal bovine serum (FBS, Bio Whittaker, Fetal Bovine Serum, South American origin). The cells were incubated at a temperature of 37°C and in the presence of 5% CO₂. Cells intended for experiments were trypsinized (Trypsin-EDTA solution, T4049, Sigma-Aldrich) and then rinsed with PBS.

Photodynamic therapy

The photosensitizer used in the therapy was Photofrin® (Ph, QLT Phototherapeutics, Inc. Vancouver, Canada). Cells were incubated for 18 h in darkness with 20 µg/ml Ph in DMEM. Next for 10 minutes it was irradiated with light with power of 10 mW/cm² using a lamp (OPTEL, Opole, Poland) with a red filter (632.8 nm) [15, 16]. Cells were again incubated in the same conditions for 3, 6 or 24 h.

Proliferative test

Cell survival was assessed by checking cellular mitochondrial activity. Metabolic activity was evaluated using the tetrazolium salt reduction test in cell mitochondria (MTT Assay, Sigma Chemical Co.; 71K8409, In Vitro Toxicology Assay). Mitochondrial activity of living cells was examined in 96-well plates. 3 × 10⁴ cells were placed in each well. The measurements were made on a Multiskan MS microplate reader (Labsystem) at wavelength 570 nm. Results are shown as percentage of control.

Localization of photosensitizer and mitochondria

Cells were incubated for 4 hours on microscope coverslips in the presence of the photosensitizer. Cells were fixed with 4% paraformaldehyde, and then rinsed with PBS. To stain the mitochondria, cells were incubated with 100 nmol/l Mito-Tracker Green (Molecular Probes, Eugene, OR, a fluorescent dye which stains mitochondria green) for 10 minutes. Such prepared preparations were evaluated using a confocal microscope (LSM510 Meta, Zeiss). For intracellular distribution of photosensitizer a filter with an excitation wavelength $\lambda = 405\text{--}753$ nm was applied. To illustrate the cellular mitochondria, a filter with an excitation wavelength $\lambda = 488$ nm was used.

Results

Proliferative test

There were observed clear differences between the survival of irradiated samples with Photofrin®, and the survival of non-irradiated samples (Fig. 1). The viability of MEWO cell

line after PDT and 24 h incubation reached 22% and for Me45 cell line only 19%. After the same time of incubation, but without irradiation, 83% of MEWO and 65% of Me45 cells survived. The experiment showed that the applied photodynamic method is especially cytotoxic to the primary cell line (Me45). For each time of incubation differences in viability of photodynamically treated cells and non-irradiated samples were significant.

Localization of photosensitizer and mitochondria

In MEWO cell line the photosensitizer evenly within the cellular cytoplasm and mitochondria, whereas in Me45 cell line it accumulated primarily in mitochondrial membranes (Figs. 2, 3).

Discussion

Results presented in this paper revealed that applied PDT is cytotoxic to tested human melanoma cells. Cell survival decreased with incubation time after irradiation for both treated cell lines. Particularly sensitive to the applied therapy were primary melanoma cells (MEWO). In both cell lines the localization of Photofrin was observed mainly in mitochondrial membrane, which may lead to induction of intracellular disorders, release of apoptogenic proteins and finally to cell apoptosis [17]. Other researchers have also shown that the application of PDT in melanoma treatment is effective. The authors observed DNA damage in G361 cell line after PDT with porphyrin derivatives, which provoked apoptotic death of malignant melanoma cells [18]. Nowak-Sliwińska *et al.* proved that Verteporfin and Photofrin used in PDT are highly effective in mouse melanoma S91/13 Cloudman cells [19]. The researchers observed increasing levels of singlet oxygen in cells, accompanied by a significant decrease in cell survival. Chen *et al.* found that PDT with methylene blue (MB) caused oxidative stress, which plays an important role in initiating cell death [20]. Our studies show that PDT with

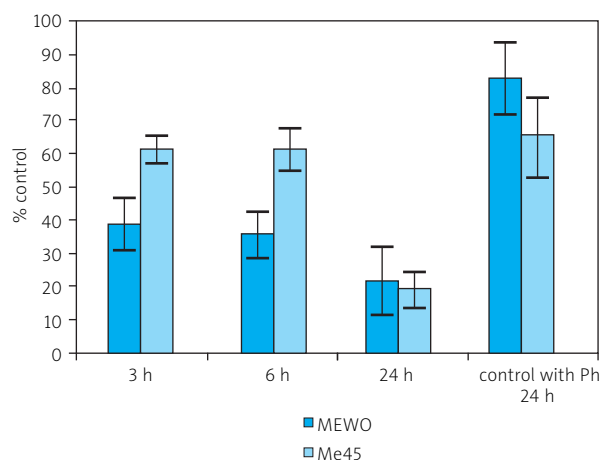


Fig. 1. Evaluation of mitochondria as an indicator of cell survival and ME45 MEWO after photodynamic reaction using 20 mg/ml of Ph at a dose of light with a power of 10 mW/cm² for 3, 6 and 24 hours of incubation after irradiation. Results expressed as mean ± SD

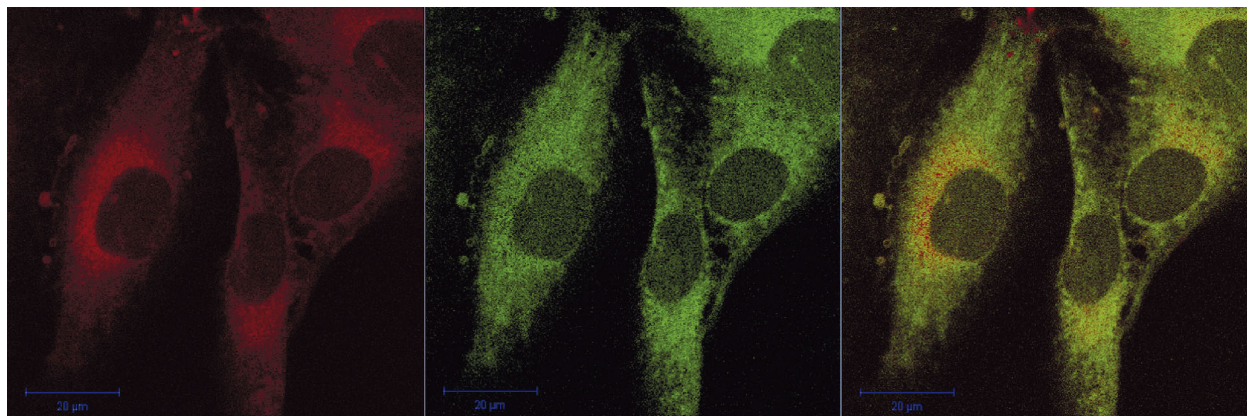


Fig. 2. Distribution of Ph in MEWO recorded in cells after 4 h incubation with photosensitizer: A – location of Photofrin®; B – cell mitochondria marker MitoTracker stained MEWO green; C – superimposition of images A and B

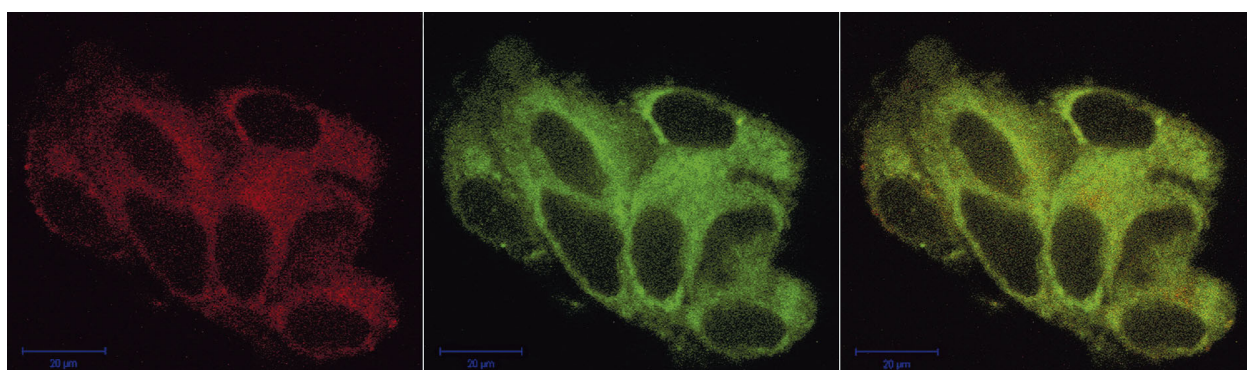


Fig. 3. Distribution of Ph in Me45 recorded in cells after 4 h incubation with photosensitizer: A – location of Photofrin®; B – cell mitochondria marker MitoTracker stained Me45 green; C – superimposition of images A and B

Photofrin® is a promising technique that can be combined with chemotherapy or radiotherapy, especially in the early stage of melanoma. It can also be a perfect adjuvant therapy after resection of malignant lesions [21]. In conclusion, PDT opens new non-invasive possibilities of melanoma treatment with application of modern developments in molecular biology, chemical synthesis of compounds and optical physics.

Acknowledgments

The experiments were funded by a PhD grant from the Ministry of Science and Higher Education, no. 5409/B/P01/2011/40 (project leader: PhD. Jolanta Saczko), and funds of a research project for young scientists of the Medical University of Wrocław: grant for young scientists no. PBmn1 (project leader: PhD. Jolanta Saczko).

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Address for correspondence**Anna Choromańska**

Department of Medical Biochemistry
Wrocław Medical University
Chatubińskiego 10
50-368 Wrocław, Poland
tel. +48 71 784 13 87
e-mail: awawrzuta@gmail.com

Submitted: 10.02.2011**Accepted:** 7.02.2012