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The influence of photodynamic therapy on apoptosis in human melanoma cell line

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Abstract: Melanoma is the most severe of all skin cancers as it may grow rapidly and metastasize. The application of photodynamic therapy (PDT) opens new perspectives in treatment of this cancer. Numerous studies suggest that the exposure of tumor cells to PDT can lead to cell death *via* two separate processes: apoptosis or necrosis. The aim of this study was to assess *in vitro* photodynamic therapy which induces apoptosis in the human Beidegröm Melanoma (BM) cell line, using neutral comet assay. The cells were incubated with Photofrin II (15 μg/ml and 30 μg/ml) 4 h before and 3 h after irradiation for 5 or 10 min with the light intensity of 10 mW/cm^2, using a lamp with red filter (632.8 nm). The percentage of apoptotic cells was significantly higher after PDT comparing to control cells. We observed 25% and 70% of apoptotic cells after shorter irradiation and treatment with 15 μg/ml and 30 μg/ml of Ph II, respectively. After longer irradiation, the respective values were 71.9% and 90%. The results suggest that induction of apoptosis is an important determinant of photodynamic sensitivity in the studied cell line and that some types of DNA damage are dependent on photosensitizer concentration and time of irradiation.

Key words: Photodynamic therapy - Neutral comet assay - Photofrin II - Melanoma - Cell culture

Introduction

Melanoma has been for a long time one of the most dangerous cancers in Poland. Although it accounts for only about 4% of all skin cancer cases, this tumor causes most skin cancer-related deaths, being the most severe of all skin cancers because of its ability to grow rapidly and to metastasize. Melanoma can occur in all regions of the skin, however, it is more common in some regions *e.g.* in males on the trunk, while in females on the legs. Rarely melanomas can occur in eyes, in the nervous system and in mucous membranes. There is no fully effective treatment, and 95% of melanoma cases are treated first with surgery. Other treatments include: chemotherapy, immunotherapy, radiation therapy and combination of the three [3]. The application of photodynamic therapy (PDT) opens new perspectives in the therapy of this cancer. PDT is an effective local cancer treatment that induces cytotoxicity through intracellular generation of reactive oxygen species (ROS). The interaction between the excited photosensitizer and molecular oxygen yields singlet oxygen ($^1O_2$) as well as other ROS to induce cell death [6, 18]. The mechanism of photodynamic reaction in tissues has not been fully elucidated. The course of changes observed in cells under the influence of PDT can be different, depending on the oxygen accessibility, the type of photosensitizer and its concentration, as well as the intensity of light. The disintegration of cellular structures and modulation of genetic information induced by PDT directs cancer cells to a death pathway [2, 15, 16]. Numerous studies suggest that the exposure of tumor cells to PDT can lead to cell death *via* two separate processes: apoptosis or necrosis. In contrast to necrosis, apoptosis is an energy dependent, distinct form of cell death that follows a sequence of genetically programmed events and proceeds without inflammation [1, 5].

One of several methods of apoptosis detection is the neutral comet assay which uses single cell gel electrophoresis to detect DNA strand breaks in individual mammalian cells. The comet assay is a relatively simple technique that allows to determine not only the percentage of apoptotic nuclei, but also the extent of genotoxic damage induced by an apoptotic trigger [12]. In its original form, cells embedded in agarose on microscope

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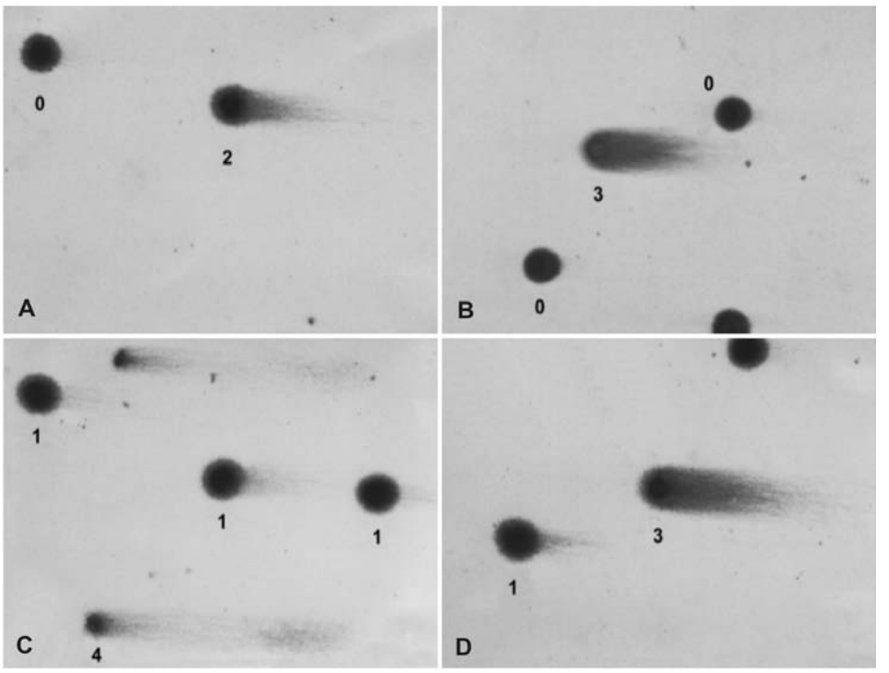

Fig. 1. Different kinds of DNA damage visualized by comet assay. **A** - comet type 0 and 2; **B** - comet type 0 and 3; **C** - comet type 1 and 4; **D** - comet type 1 and 3. Type 0 - size of the head as normal nucleus size, tail absent; type 1 - size of the head as normal nucleus size, tail size less than normal nucleus size; type 2 - size of the head as normal nucleus size, tail size about 1-2 times normal nucleus size; type 3 - size of the head as normal nucleus size, tail size about 2-3 times normal nucleus size; type 4 - size of the head less than half size of normal nucleus, tail size more than 3 times normal nucleus size.

slides are lysed and subjected to electrophoresis under neutral conditions, enabling the detection of DNA double strand breaks [4, 6].

This assay can detect various forms of DNA strand breaks depending on the pH of electrophoresis [6]. Under alkaline conditions (pH 13), it detects single-strand breaks, double strand breaks, excision repair sites, and alkaline-labile sites. Under neutral conditions, it mainly detects double strand DNA breaks [9, 13] and is therefore considered to be suitable for detection of apoptosis [21].

The objective of the present study was to assess *in vitro* photodynamic therapy which induces apoptosis in human Beidegröm Melanoma (BM) cell line.

Materials and methods

Cell culture. Beidegröm Melanoma (BM) cell line, established from human melanoma, was obtained from the Institute of Immunology and Experimental Therapy, Polish Academy of Sciences, Wroclaw. The cells were grown in MEM medium (Sigma) with addition of 10% foetal bovine serum (Biowhittaker). For experiments, the cells were removed by trypsinization, and washed with PBS. The cells were maintained in a humidified atmosphere at 37˚C and 5% CO_2.

Photodynamic treatment. The Photofrin II (Ph II, QLT Phototherapeutics, Inc. Vancouver, Canada), a photosensitizer that has been accepted for clinical studies, was used for the photodynamic therapy *in vitro*. The cells were incubated for 4 h, in the dark, with two doses: either 15 μg/ml or 30 μg/ml of Ph II in MEM. Then they were irradiated with the light intensity of 10 mW/cm^2 using a lamp (OPTEL, Opole, Poland) with polarized light and red filter (632.8 nm) and incubated again for 3 h at 37˚C and 5% CO_2 in MEM. Two irradiation times: 5 min and 10 min, equivalent to light doses of 3 J/cm^2 and 6 J/cm^2, respectively, were tested.

Neutral comet assay. For detection of DNA fragmentation associated with apoptosis, neutral comet assay method described by Collins [6] was used. BM cells, at a concentration of 1×10^5/ml, were mixed with low temperature melting agarose (Sigma) at ratio 1:10 (v/v) and spread on a slide. Slides were submerged in precooled lytic solution (2.5 M NaCl, 100 mM EDTA, pH 10, 10 mM Tris base and 1% Triton X-100) at 4˚C for 60 min. After lysis and rinsing, 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 [10]. For scoring the comet patterns, 100-200 nuclei from each slide were assessed.

To rank apoptotic comets, we followed the method developed by Collins [6]. We were able to distinguish five types of comets. Figure 1 shows the detailed categorization method used for evaluation of our samples. For comparison of comet scores we used χ^2 test. The results turned out to be statistically significant, after checking a hypothesis that the type of comet is dependent on PDT with the probability of the first kind error $\alpha = 0.05$.

Results and discussion

In order to determine the phototoxicity of Photofrin II-mediated PDT, fragmentation of DNA was evaluated. Neutral comet assay detects only double-stranded DNA breakage, characteristic of apoptotic death [14, 21]. Under alkaline conditions, DNA denaturation and separation of double-stranded DNA occur, in contrast to neutral conditions, where during DNA uncoiling and following electrophoresis, the denaturation of DNA does not take place. Therefore, the nuclei with single-stranded DNA breaks are detected in alkaline method as comets. However, such nuclei are not observed as comets under neutral conditions, because each damaged strand is still associated with undamaged one and is detected as comet type 0 (Fig. 1A, B). The apoptotic cell

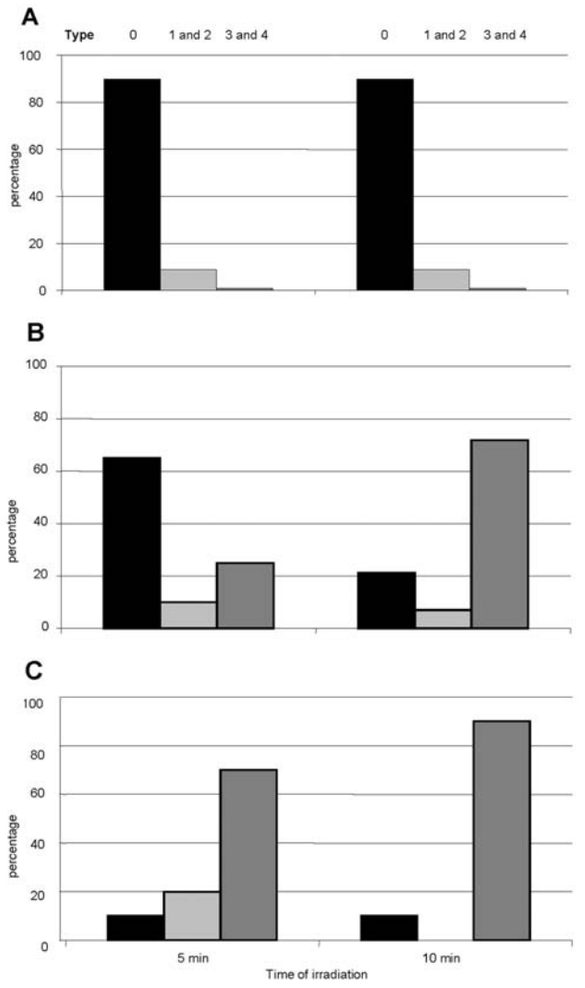

Fig. 2. The percentage of nuclei with DNA damage in BM cells induced by Photofrin II-mediated PDT. **A** - control (not irradiated), **B** - 15 μg/ml Ph II, **C** - 30 μg/ml Ph II. Left histograms in B and C - 5 min irradiation; right histograms in B and C - 10 min irradiation.

nuclei are categorized as type 3 or 4 comets (Fig. 1B-D). Comets of type 1 and 2 (Fig. 1A, C, D) reflect undefined DNA changes.

The results are presented in Figure 2. The percentage of apoptotic cells was significantly higher for cells after PDT than for control cells (Fig. 2A). We observed 25% and 70% of apoptotic cells after 3 J/cm^2 irradiation and treatment with 15 μg/ml and 30 μg/ml of Ph II, respectively (Fig. 2B). After 6 J/cm^2 irradiation, the respective values were 71.9% and 90% (Fig. 2C).

The efficiency of photodynamic therapy in the treatment of malignant melanoma is not well defined. Most studies on PDT effects were focused on those that lead to cell death [19]. Cell death caused by PDT (through the generation of ROS) can occur either by apoptosis as interphase death or as secondary event following mitosis or by necrosis, depending on the cell type, oxygen level, concentration and intracellular localization of the sensitizers, and the light dose [1, 7, 20]. The early studies concerning the influence PDT on melanoma were performed on murine malignant melanoma *in vitro* and *in vivo* using aluminum phtalocyanine (AlpcS4) [2, 8]. PDT was found to have significant effects in this experimental melanoma and apoptosis induced by PDT seemed to play an important role in the photodynamic treatment efficacy [2].

The present study shows that Photofrin II PDT induces cell death in BM line mainly through apoptosis. Gupta *et al.* [7] observed differences in sensitivity to hematoporphyrin derivative HpD (photoscan-3; PS-3) PDT between human glioma (BMG-1) cell line carrying wild type tumor suppressor gene p53 and a human squamous carcinoma cell line (4451) with mutated p53. They studied PDT-induced apoptosis using alternative methods (flow cytometric analysis of DNA content, phosphatidylserine externalization, light-scatter analysis and fluorescence microscopy). They observed that PS-3 PDT induced a significantly higher level of apoptosis in human glioma (BMG-1) cells as compared to squamous carcinoma (4451) cells. This was dependent on the concentration of PS-3 as well as post-irradiation time in both cell lines. At 2.5 μg/ml of PS-3, the fraction of BMG-1 cells undergoing apoptosis (60%) was nearly 6-fold higher than that of 4451 cells (10%). The increase in PS-3 concentration to 5 μg/ml increased apoptosis in BMG-1 cells, however, further increase in PS-3 concentration to 10 μg/ml caused decrease in the percentage of apoptotic cells in BMG-1 line but increase in 4451 line [7].

In this study we demonstrated that BM cell sensitivity to Ph II PDT is dependent on Ph II concentration and light dose. In contrast to studies of Gupta *et al*. [7], the highest percentage of apoptotic cells was achieved at 10 μg/ml Ph II and 10 min of irradiation. Similar results were obtained in human melanoma cells and a new silicon phtalocyanine photosensitizer which has the property of triggering cell apoptosis mediated by mitochondria [2]. Nagata *et al.* [11] performed studies concerning phototoxic effect and cell death modes of human malignant melanoma cells following photodynamic therapy with ATX-S10(Na), an amphifilic photosensitizer and demonstrated higher phototoxicity with higher dye and light doses. Most of the dead cells appeared apoptotic after dye and irradiation doses that induced less than 70% cytotoxicity. In contrast, most of cells appeared necrotic after doses that induced 99% cytotoxicity. These results seem unusual, as the primary sites of ATX-S10(Na) accumulation were lysosomes, not mitochon- dria. In our studies we used Ph II as a photosensitizer which accumulates mainly in the mitochondrial membranes [11]. This leads to disturbances of mitochondrial transmembrane potential and finally to apoptotic cell death.

The photodynamic therapy was successfully applied in clinical settings to destroy neoplasms, but the efficacy of such treatment is dependent on the type of neoplasm and the photosensitizer. The investigations concerning the influence of PDT on melanoma *in vivo*, using chlorin e(6), demonstrated complete regression in all skin melanoma metastases with no recurrence during the study period [16]. PDT of melanoma with porphyrin and porphyrin derivatives is effective and well tolerated by patients [17]. The present results show that higher sensitivity to Ph II PDT in BM cells is manifested by higher level of apoptosis and suggest that induction of apoptosis is an important determinant of photodynamic sensitivity in this malignant cell line. The application of comet assay to study the influence of Photofrin II PDT on BM cells demonstrated that some types of DNA damage depend on photosensitizer concentration, as well as on time and dose of irradiation.

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