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Photofrin II-based photosensitization of human ovarian clear-cell carcinoma cell line (OvBH-1)

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Abstract:
The cytotoxic effect of photodynamic therapy (PDT) on ovarian clear-cell carcinoma cell line, OvBH-1 was achieved using different methods.

No clear morphological changes in OvBH-1 after PDT were observed, whereas PDT-treated MCF-7 breast carcinoma cells demonstrated morphological features of apoptosis. After PDT, OvBH-1 pattern of p53 expression remained unchanged, but MCF-7 cells revealed nuclear/cytoplasmic p53 expression. After PDT, a weak Bax protein induction was found in both PDT-treated cell lines. Bcl-2 expression in PDT treated OvBH-1 cells remained unchanged, while it was markedly decreased in PDT treated MCF-7 cell line. MCF-7 cells expressed high level of neoepitope cytokeratin 18 (CK 18) (M30), whereas low expression of neoepitope cytokeratin 18 was found in OvBH-1, after PDT. The photofrin II PDT resulted in a significant reduction of the activity of mitochondrial enzymes in MCF-7 cells , as compared with that of OvBH-1 cells. The photofrin II effect, measured as a percentage of cells with high mitochondrial activity observed in MCF-7 cells at 3 and 6 h after PDT was low (6 and 3%, respectively), whereas in these conditions as many as 28.9% (at 3 h) and 30.42% (at 6 h) of OvBH-1 cells showed significantly higher activity of mitochondrial enzymes ($p < 0.001$). It is worth underlining that both methods, alkaline comet assay and flow cytometry, revealed that the majority of OvBH-1 cells (75–95.4%) were dead with the signs of necrosis, while in MCF-7 cells most of them (80–92%) died by apoptosis. Our *in vitro* preliminary results indicate that PDT causes non-apoptotic death in OvBH-1 cell line.

Key words:
OvBH-1, MCF-7 cell lines, PDT, apoptosis, necrosis

Introduction

Ovarian cancer, after the breast cancer, is the second most common female cancer in Poland. It is a pathologically and clinically heterogeneous disease with variable prognosis. Because of its invasive growth and metastases, ovarian cancer has the highest mortality rate among gynecological malignancies. Chemotherapy is the most commonly used treatment of patients with ovarian cancer, but approximately 30% of advanced epithelial ovarian cancer cases are drug-resistant prior to or develop resistance during first-line chemotherapy, and nearly 50% of originally responsive patients develop chemoresistance during the whole course of treatment [14, 17]. Another problem associated with the use of chemotherapy in clinical practice is the fact that patients having the same histopathological type of tumor and grade of differentiation display a variety of responses to applied treatment [18]. Therefore, one of the most important point is to develop new strategies in the ovarian carcinoma treatment. One of the promising approaches is photodynamic therapy (PDT) [7, 30]. PDT is already accepted as a treatment of patients with superficially located tumors, accessible for applied light source, e.g. in urinary bladder, endobronchial, esophageal, colon and breast cancers [6, 7, 24].

Most anticancer agents used in the treatment of ovarian cancer may induce tumor regression by apoptosis [14]. It is well documented that one of the major reasons for the lack of response to therapy may be a defect in apoptotic cell death executive mechanisms [29]. Apoptotic process is regulated by some protooncogenes, apoptosis regulating genes, particularly those promoting apoptosis (Bax, Bad, Bcl-Xs) as well as antiapoptopic (Bcl-2, Bcl-XL) and tumor-suppressor genes [13, 22, 23, 25]. Among the latter, the p53 gene product seems to have a crucial role in the control of apoptotic pathway by Bax gene transactivation [13, 25]. Hence, it is known that p53 gene is mutated in about 50% of ovarian carcinomas and there is a probability that mutated p53 protein lacks the ability to transactivate the genes that are involved in the regulation of apoptosis [21, 29]. Individual data indicated that beside the Bax protein expression, cytokeratins, in particular cytokeratin 18, are early affected in the process of apoptosis [15]. The neoepitope derives from cleavage of cytokeratin 18 by caspase 8, which is recognized by the specific monoclonal antibody M30 CytoDEATH. This neoepitope is not visible in nonapoptotic cells and in native cytokeratin 18 of normal cells, but are present in apoptotic tumor cells [15].

The PDT therapy involves administration of a drug, most often a porphyrin derivative, and subsequent irradiation with light of a proper wavelength. This combined treatment results in generation of highly reactive oxygen species (ROS), like singlet molecular oxygen, hydroxyl radicals and/or superoxide anions [17, 24]. Singlet oxygen is recognized to represent the main and most important factor affecting cellular structures through lipid peroxidation. *In vivo*, apart from a direct tumor cell destruction, PDT results in changes in blood vessels, e.g. in vasoconstriction, platelet aggregation and leakages. Hence, the mechanism of cell damage induced by photodynamic effect is different from cytotoxic effects induced by the majority of chemotherapeutic agents and it is possible that PDT may be effective against tumor cells expressing multidrug resistance for classical chemotherapy. It has been shown that PDT may induce cell death *via* apoptosis as well as necrosis pathways [16, 19]. Response to photodynamic treatment is dependent on the type of photosensitizer used in a study, irradiation conditions, the oxygenation and the type of cells involved. Subcellular localization of the photosensitizer is of special importance, since it determines localization of the primary damage [30]. To our knowledge, limited data are available concerning the PDT use in ovarian carcinoma, and there are no data defining PDT responsiveness as a function of p53 status in ovarian clear cell carcinoma cells. Taking into account the worst prognosis of ovarian clear cell carcinoma among five different histological types of ovarian carcinoma, it was interesting to estimate the PDT effect on ovarian clear cell carcinoma cells.

The aim of this study was to evaluate the cytotoxic effect of PDT on ovarian clear cell carcinoma cell line OvBH-1, overexpressing p53 protein.

Materials and Methods

Cell lines

The OvBH-1 cell line was established at the Department of Clinical Immunology, Wrocław Medical University, Poland, from ascitic fluid cells of a 54-years

old woman with ovarian clear cell adenocarcinoma. The morphological and immunophenotypic characteristics as well as temperature-sensitive behavior of OvBH-1 cells was described earlier [4]. A lot of studies concerning PDT were performed on the well-characterized cell line MCF-7 [9], so the human mammary adenocarcinoma cell line MCF-7 (ATCC, HTB-22) was used as a control. Both cell lines were maintained in growth medium: Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (both from Gibco) in 25 cm^2 TC flasks (Falcon) at 37°C, in a 5% CO_2 humidified atmosphere. Exponentially growing cells were aliquoted into 96-well TC plates (Falcon) at the density of 3×10^3 cells/well and allowed to grow for 2 days before being used for the experiments.

Photosensitizer

Photofrin II was a kind gift from Professor R. Bonnett from University of London. Photofrin II crystals were dissolved in growth medium at pH = 7.1–7.2. The stock solution was kept in dark at temperature below 0°C. The working concentration of the photosensitizer was 20 μg/ml.

Light source

Penta Lamps, Teclas (CH) were used as a light source. The light was passed through a 5 mm fiber to the illuminated area. The fluence rate was kept at 120 mW/sq cm and established wavelength was 630 ± 20 nm. The total light dose was 21.6 J/sq.cm obtained after 6 min of irradiation.

PDT

The cells were incubated with Photofrin II for 18 h before irradiation. After irradiation, cells were examined at 3 and 6 h time points.

Morphology of cells

Cytospin preparations were made of cells from both OvBH-1 and MCF-7 cell lines before and after PDT treatment and fixed in cold acetone for 10 min, stained with hematoxylin and eosin, and evaluated by light microscopy.

Photofrin II cytotoxicity evaluation

Cells were incubated with the appropriate photofrin II containing media for 18 h before irradiation. After PDT, cells were removed by trypsinization, diluted in PBS and plated into 96-well microtiter plates. The activity of mitochondrial enzymes was determined by MTT assay that measures the reduction of 3-(4,5-dimethylthiazol-2-yl)-diphenyltetrazolium bromide substrate to a dark-blue formazan product by mitochondrial dehydrogenases in living cells (Sigma Chemical Co., 71K8409, *in vitro* Toxicology Assay). Aliquots (90 μl) of cell suspensions and 0.5 mg of MTT (10 μl) were added to each well and incubated for 2 h. The supernatant was removed by aspiration and 100 μl of dimethyl sulfoxide (DMSO) was added to each well. The microtiter plate was subjected to shaking to dissolve the formazan. The absorbance of each well was determined using the multiwell scanning spectrophotometer at 570 nm (Multiscan MS microplate reader).

Antibodies

The following monoclonal antibodies were used: anti-p53 protein clone DO-7, anti-Bcl-2 oncoprotein clone bcl-2/100/D5 (both Novocastra, Newcastle, UK), anti-Bax protein, clone I-19 (Santa Cruz, USA), anti caspase cleaved product of cytokeratin 18, clone M30 (M30 CytoDEATH) (Boehringer Mannheim, Germany) diluted 1:25, 1:80, 1:100, 1:50 respectively.

Immunohistochemical staining

Peroxidase-antiperoxidase tests were performed on cytospin preparations of OvBH-1 and MCF-7 cells, before and after PDT. After inhibition of endogenous peroxidase with periodic acid (2.28%) and sodium borohydride (0.02%) and 30 min of incubation with normal rabbit serum, the cell cytospin preparations were treated with primary antibodies against the p53, Bax, Bcl-2, and neoepitope cytokeratin18 (M30). The replacement of the primary antibodies by 0.1 M Tris-buffer, pH = 7.4 served as a negative control. After 60 min of incubation with primary antibodies, peroxidase-conjugated rabbit anti-mouse IgG (Dako, Copenhagen, Denmark) was applied for 30 min. Following washing with 0.1 M Tris-buffer, pH = 7.4, the preparations were treated with peroxidase-conjugated swine anti-rabbit IgG (Dako) and the visualization

was carried out with 3,3'- diaminobenzidine (Sigma, St, Louis, MO) as a chromogen. For microscopic evaluation, the preparations were counterstained with hematoxylin and mounted. Immunohistochemical staining was evaluated using a Olympus BH microscope (Olympus, Japan). Stained cells were quantified by counting 700 cells in randomly selected fields. The result was judged positive if color reaction was observed in more than 5% of cells. The intensity of immunohistochemical staining was evaluated as: (–) negative, (+) weak, (++) moderate and (+++) strong. All experiments were repeated twice.

Alkaline comet assay

For detection of DNA fragmentation associated with apoptosis the alkaline comet assay method described by Ashby et al. [2] was used. OvBH-1 and MCF-7 cells, at a density of 1×10^5/ml, were mixed with low temperature melting agarose (Sigma) at 1:10 (v/v) ratio and spread on a slide. Slides were submerged in precooled lysis solution (1.2 M NaCl, 30 mM NaOH, 2 mM EDTA, 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 [20]. For scoring the comet pattern, 100–200 nuclei from each slide were evaluated.

Determining of apoptotic cell population by flow cytometry

Cells (2×10^5/sample) were washed once with PBS, resuspended in 0.5 ml of ice-cold 70% ethanol and incubated on ice for > 2 h. Then, the cells were washed 3 times with PBS, resuspended in 1 ml of RNAse (Sigma) solution in PBS (200 µg/ml) and incubated for 30 min at 37°C. Cellular DNA was stained with propidium iodide (PI; 40 µg/ml). Cells were analyzed with FasCalibur flow cytometer (Becton Dickinson BD, Mountain View, CA) equipped with 488 nm argon laser. Ten thousand events per sample were registered using CellQuest acquisition software and further analyzed using the ModFit LT software (both Becton Dickinson).

Statistical analysis

Differences in mean values between untreated OvBH-1 and MCF-7 cell lines (control groups) and after PDT at 3 and 6 h (separately for each cell line; OvBH-1 and MCF-7) and between OvBH-1 and MCF-7 cell lines before and after PDT were analyzed using one-way ANOVA and planned comparisons linear contrast method. P value less than 0.05 was considered statistically significant. Statistical analysis has been done by dr Leszek Noga from Department of Pathophysiology, Medical University, Wrocław, Poland.

Results

Morphological features of OvBH-1 and MCF-7 cells before and after PDT

The cytomorphological features of OvBH-1 and MCF-7 cells before and after PDT at different time points are presented in Table 1 and Figure 1A, 1B and Figure 2A, 2B. The distinct morphological changes, i.e. vacuoles in cytoplasm appeared 3 h after PDT in approximately 20% of OvBH-1 cells and after 6 h, vacuoles were found in the cytoplasm of 60% of cells, accompanied with changes in shape and size of nuclei. However, these changes were not apoptosis-specific, whereas 3 h after PDT MCF-7 cells displayed obvious morphological features of apoptosis such as cell shrinkage, nuclear condensation and the presence of apoptotic bodies.

Immunohistochemical evaluation of analyzed proteins

In untreated OvBH-1 cells p53 was strongly expressed in 90% of cell nuclei, whereas MCF-7 cells were found to be negative (less than 5% of positive cells) (Tab. 2). After PDT, intense nuclear p53 staining was still visible in OvBH-1 cells (Fig. 1C), while in MCF-7 cells the percentage of p53 positive cells significantly increased up to 60%. The majority of MCF-7 cells showed the cytoplasmic localization of p53 with diffuse staining of nuclei (Fig. 2C).

Untreated control cells of both used cell lines were negative (less than 5% of positive cells) for proapoptotic Bax gene product. After PDT, weak Bax protein induction was visible in both PDT-treated cell lines at

Tab. 1. Analysis of morphological changes in cells from OvBH-1 and MCF-7 cell lines before and after Photofrin II-mediated photodynamic therapy (PDT)

Morphological Features		Before treatment		After PDT treatment			
				3 hours		6 hours	
		OvBH-1	MCF-7	OvBH-1	MCF-7	OvBH-1	MCF-7
Cells	Size	+++	++	++	+/++	++	+/++
	Shape	++	++	–/++	–/++	–	–
	Borders	–	+	–/+	–/+	–/+	–
	Apoptosis:						
	Nuclei condensation (nc)	–	–	–	+	–	++
	Apoptotic bodies (ab)	–	–	–	+	–	+
Nucleus	Shape	++	++	–/++	–/+	–/+	–/+
	Nucleoli	++	–	++	–	++	–
Cytoplasm	Size	+	++	+/++	+/++	+/++	+/++
	Vacuoles	+	–	+	–	++	–

Size: + small, ++ moderate, +++ large; Shape: – irregular, + oval, ++ round; Borders: – indistinct, + distinct; Nucleoli: – not found, + single, ++ numerous; Vacuoles: – not found, + single, ++ numerous; Nuclei condensation (nc) or apoptotic; bodies (ab): – not found, + single ab or nuclei, ++ numerous ab or nuclei

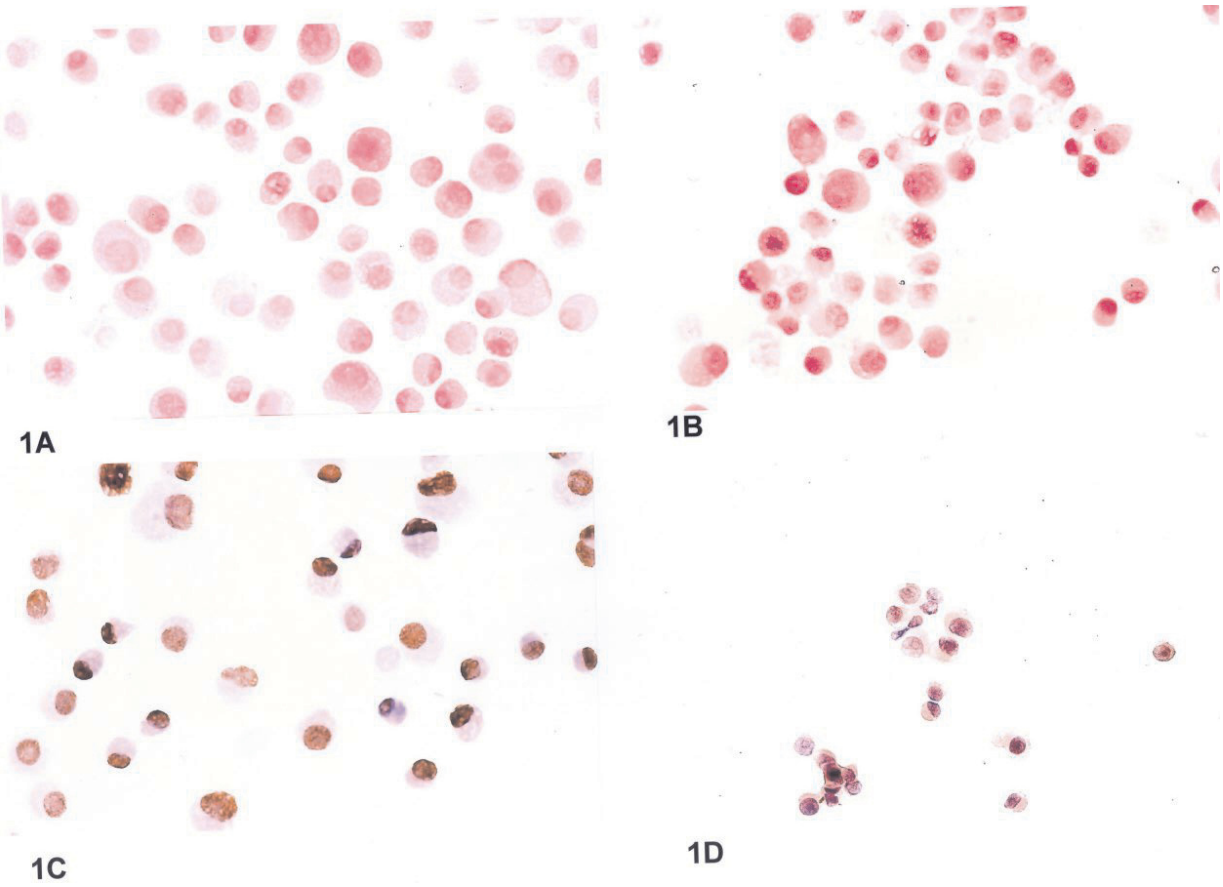

Fig. 1. Morphological features of OvBH-1 cells (**A**) before and (**B**) 6 h after PDT, (**C**) immunohistochemical staining with anti-p53, 6 h after PDT; (**D**) Bax expression 6 h after PDT (hematoxylin and eosin staining – 1A, B x 400; immunoperoxidase staining – 1C, D x 400)

Tab. 2. Expression of apoptosis-related proteins in ovarian carcinoma (OvBH-1) and breast carcinoma (MCF-7) cell lines before and after Photofrin II-PDT

proteins	Before PDT (% of positive cells)		After PDT (% of immunopositive cells)			
			3 hours		6 hours	
	OvBH-1	MCF-7	OvBH-1	MCF-7	OvBH-1	MCF-7
p53	90$^{\blacklozenge}$	< 2*	95$^{\blacklozenge}$	50*$^{\blacklozenge}$	95$^{\blacklozenge}$	60*$^{\blacklozenge}$
BAX	< 5	< 2*	> 5	10*	> 5	10*
Bcl-2	10$^{\blacklozenge}$	60*$^{\blacklozenge}$	10	< 5	10	10*
Cytokeratin 18 (CK 18) (M30)	< 5	< 5*	10	10	> 5$^{\blacklozenge}$	40*$^{\blacklozenge}$

The percentage of positive cells are expressed as the mean values ± SD. p-values as follows: * p = 0.001 for p53 immunostaining in untreated MCF-7 *vs.* MCF-7 cells after PDT at 3 and 6 h. $^{\blacklozenge}$p < 0.001 for comparison of p53 immunostaining in OvBH-1 *vs.* MCF-7 cells before and after PDT at 3 and 6 h. * p = 0.001 for Bax immunostaning in untreated MCF-7 cells *vs.* MCF-7 after PDT at 3 and 6 h. * p < 0.001 for Bcl-2 immunostaining in untreated MCF7 *vs.* MCf-7 cells after PDT at 3 and 6 h. $^{\blacklozenge}$p = 0.002 for comparison of Bcl-2 immunostaining in OvBH-1 *vs.* MCF-7 cells before * PDT. p = 0.01 for cytokeratin 18 (M30) immunostaining in untreated MCF-7 *vs.* MCF-7 cells at 3, and * p = 0.001 at 6 hours after PDT. $^{\blacklozenge}$p = 0.007 for comparison of cytokratin 18 immunostaining in OvBH-1 *vs.* MCF-7 cells and after PDT at 6 hours

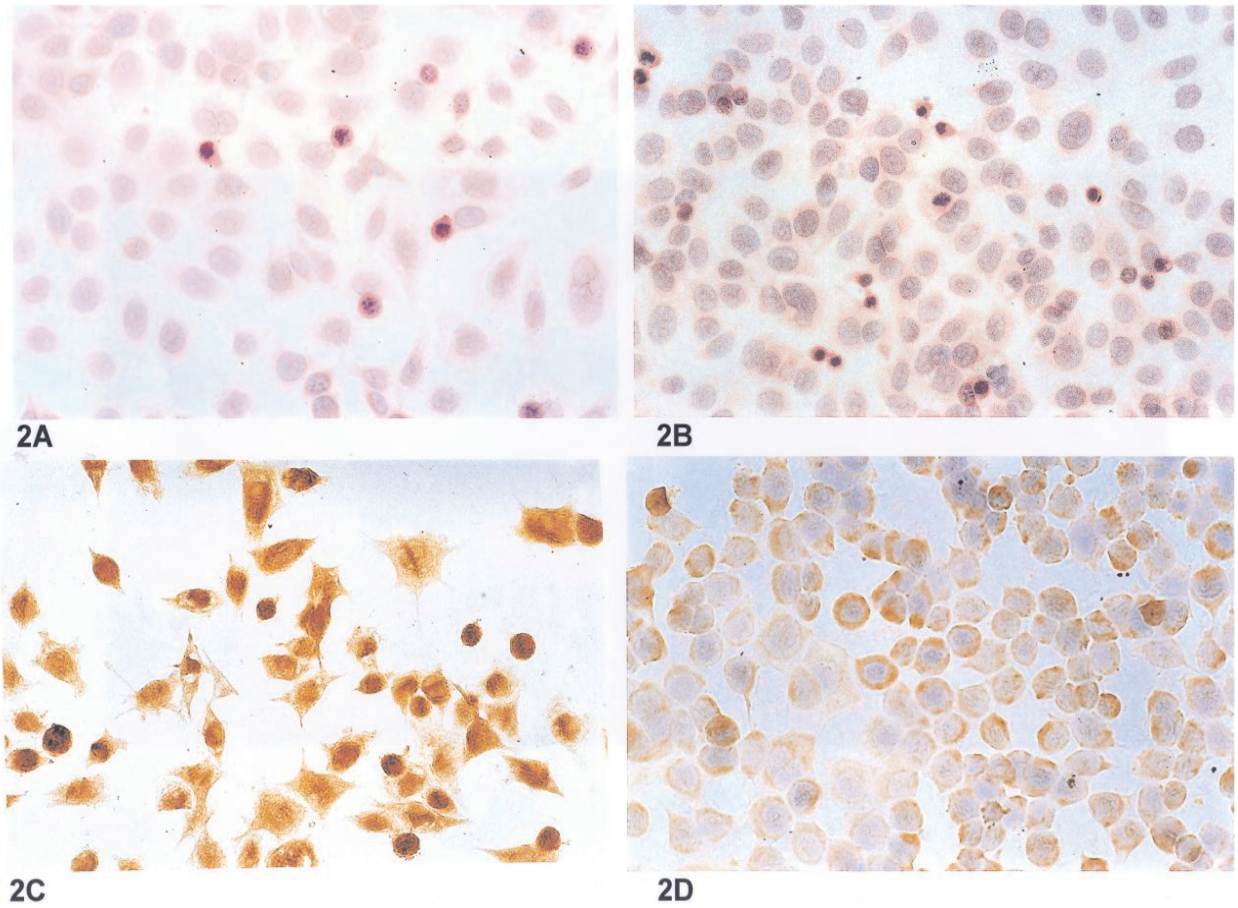

Fig. 2. Morphological features of MCF-7 cells. (**A**) before and (**B**) 6 h after PDT, (**C**) p53 protein expression 6 h after PDT; (**D**) neoepitope cytokeratin 18 (M30) expression 6 h after PDT (hematoxylin and eosin staining – 2A, B × 400; immunoperoxidase staining – 2C, D × 400)

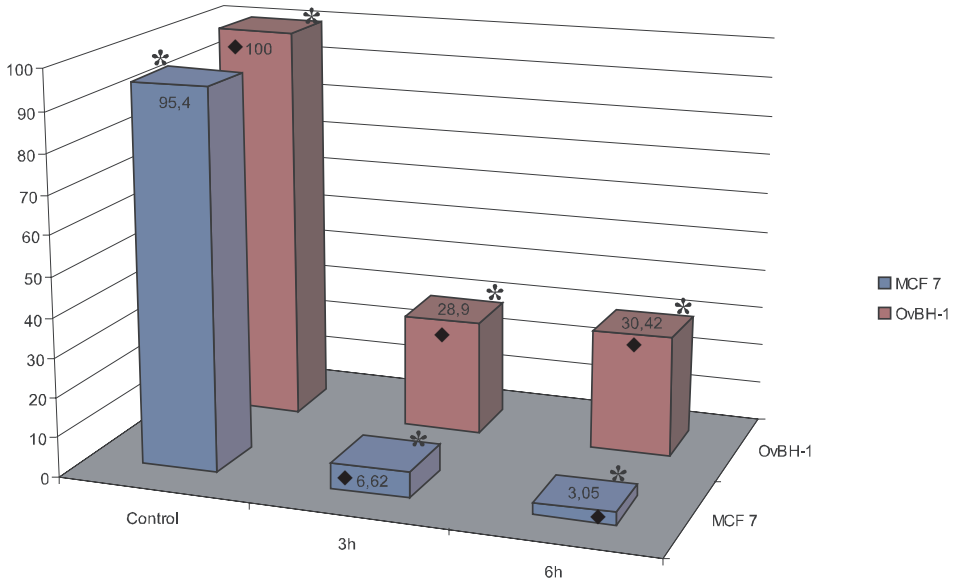

Fig. 3. Cytotoxicity test (MTT) on OvBH-1 and MCF-7 cells evaluated at 3 and 6 h after PDT. The bars represent percent cytotoxicity in OvBH-1 and MCF-7 cell lines after PDT *vs.* untreated cell lines (control). Data represent mean values ± SD. * $p < 0.001$ for untreated OvBH-1 *vs.* OvBH-1 cells after PDT at 3 and 6 h and untreated MCF-7 *vs.* MCF-7 after PDT at 3 and 6 h. ◆ $p < 0.001$ OvBH-1 cells before and after PDT at 3 and 6 h compared with respective MCF-7 cells

3- and 6-h time points (Fig. 1D). Before PDT, Bcl-2 antiapoptotic protein was highly expressed in the MCF-7 cells and decreased by up to 10% after PDT ($p < 0.001$), whereas in untreated OvBH-1 cells low Bcl-2 expression remained unchanged after PDT (Tab. 2). Before PDT, both OvBH-1 and MCF-7 cells were negative (less than 5% of positive cells) for neoepitope cytokeratin18 (M30). After PDT the number of positively stained OvBH-1 cells increased up to 10%, and up to 40% in MCF-7 cells 6 h after PDT (Fig. 2D).

MTT reduction by OvBH-1 and MCF-7 cells after Photofrin II treatment

The photofrin II PDT induced a significant reduction in the activity of mitochondrial enzymes in MCF-7 cells, as compared to OvBH-1 cells (Fig. 3). After 3 and 6 h of photofrin II PDT treatment, MCF-7 cells showed a pronounced decrease in the mitochondrial activity (to 6 and 3%, respectively). This decrease in the mitochondrial activity was lower in OvBH-1 (at 3 h – to 28.9%, at 6 h – to 30.42%). The differences between mitochondrial activity in OvBH-1 and MCF-7 cells after PDT were statistically significant ($p < 0.001$) (Fig. 3).

DNA damage evaluated by the alkaline comet assay

The alkaline comet assay results are presented in Fig. 4. Three hours after PDT, OvBH-1 cells showed up to 7.7% of apoptotic cells and up to 6.8% after 6 hours. However, the majority of cells (75–95.4%) were dead with signs of necrosis (Fig. 4). In MCF-7 cells, most of them (80–92.5%) died by apoptosis (Fig. 5). In OvBH-1 cells necrosis dominated unlike in MCF-7 cells ($p < 0.001$), whereas MCF-7 cells were significantly damaged by apoptosis ($p < 0.001$).

Determination of apoptotic cell population by flow cytometry

The percentage of apoptotic OvBH-1 and MCF-7 cells before PDT was negligible (0.04 and 0.8, respectively). After PDT, at 3 h time point OvBH-1 cell population showed no apoptosis, 6 h after PDT only 10.25% of cells became apoptotic. In contrast, the percentage of apoptotic MCF-7 cells reached 76.4% at 3 h after PDT and remained so high at 6 h time point. The observed differences between OvBH-1 and MCF-7 cells were statistically significant ($p < 0.001$) (Tab. 3).

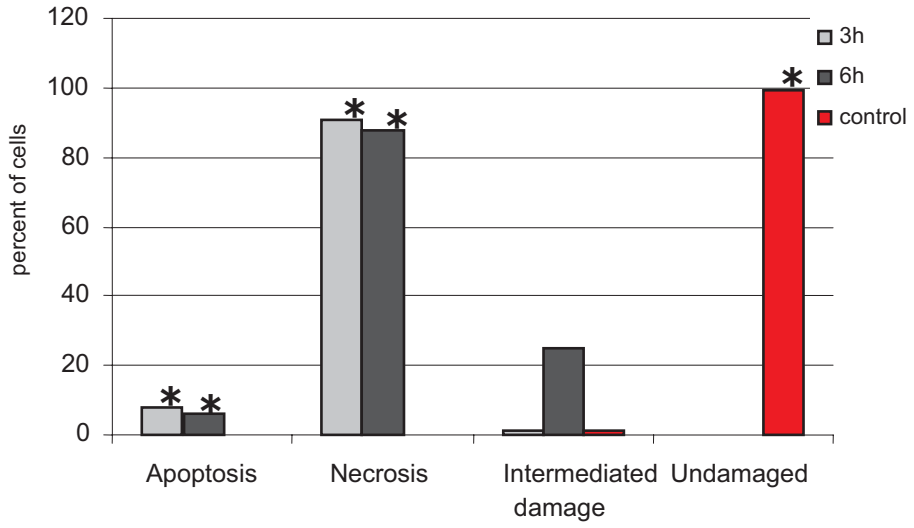

Fig. 4. DNA damage in OvBH-1 cell line evaluated by alkaline comet assay at 3 and 6 h after PDT. Data are expressed as the mean values ± SD (indicated as percentages of untreated and treated cells with necrosis or apoptosis features). * $p < 0.001$ for untreated OvBH-1 *vs.* OvBH-1 cells after PDT at 3 and 6 h (for both necrosis and apoptosis)

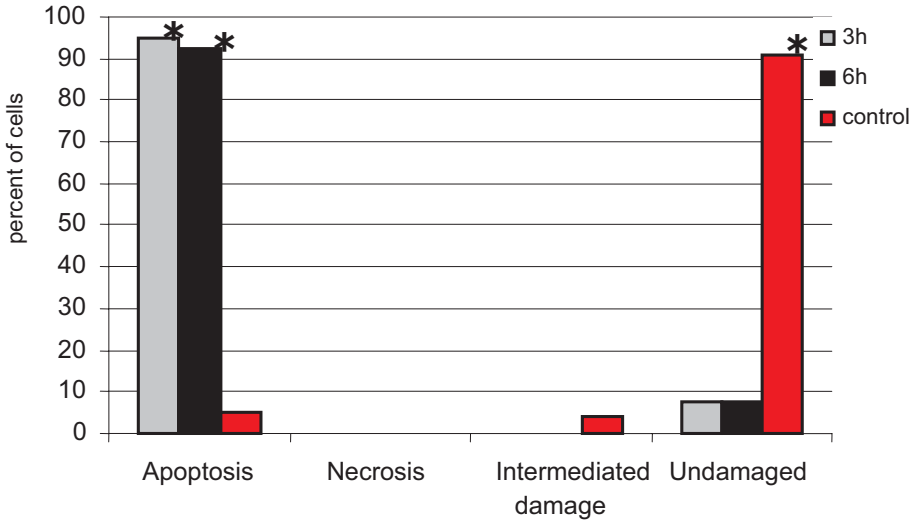

Fig. 5. DNA damage in MCF-7 cell line evaluated by alkaline comet assay at 3 and 6 h after PDT. Data are expressed as the mean values ± SD (indicated as percentages of untreated and treated cells with necrosis or apoptosis features). * $p < 0.001$ for untreated MCF-7 *vs.* MCF-7 cells after PDT at 3 and 6 h (only apoptosis)

Tab. 3. Percentage of apoptotic cells evaluated by flow cytometry

Before PDT (% of positive cells)		After PDT (% of positive cells)			
		3 hours		6 hours	
OvBH-1	MCF-7	OvBH-1	MCF-7	OvBH-1	MCF-7
0.048*◆	0.8*◆	0.3◆	76.4*◆	10.25*◆	74.1*◆

Percentage of apoptotic cells is expressed as the mean values ± SD. p values as follows: * $p < 0.001$ for untreated OvBH-1 *vs,* OvBH-1 cells after PDT at 6 h, and for untreated MCF-7 *vs.* MCF-7 cells after PDT at 3 and 6 h. ◆ $p < 0.001$ when OvBH-1 cells compared with MCF-7 cells before and after PDT at 3 and 6 h

Discussion

Following radical debulking surgery, chemotherapy plays an important role in the treatment of patients with ovarian cancer. A common clinical problem, which limits the success in treatment of ovarian carcinoma, is the development of resistance. So far, PDT is still considered to represent a new therapeutic approach in ovarian carcinoma.

Most studies on PDT effects were focused on its direct cytotoxicity, with the attention biased in favor of apoptosis, because this mechanism is mainly involved in cell death after chemotherapy or radiotherapy [13, 29]. Taking advantage of the availability of OvBH-1 cells which overexpress a stable form of p53 nuclear protein, we investigated the response of OvBH-1 cells to photodynamic therapy. We found that OvBH-1 cells demonstrated pronounced resistance to PDT-induced apoptosis, which was visualized by lack of remarkable changes in their morphological features, DNA integrity and expression of apoptosis-related proteins. Six hours after PDT, only approximately 10% of OvBH-1 cells entered apoptotic pathway. Several studies concerning sensitivity of cells to the lethal effect of PDT as well as the influence of p53 expression on the sensitivity to apoptosis induction were published [10, 19]. It has been shown that the introduction of the viral oncoprotein E6 to abrogate wild-type p53 function of MCF-7 cells did not alter their sensitivity to PDT or to other ways of apoptosis induction, thus leading to the conclusion that PDT sensitivity is not p53-dependent [11]. It has been also reported that p53-regulated genes such as GADD44 and BAX are rapidly activated during apoptosis induced by photosensitization or chemotherapeutics in human cell lines with normal p53 status [28, 32]. It cannot be excluded that the lack of apoptosis induction in our OvBH-1 cells resulted in a block of expression of p53-associated genes. However, we found that the expression of Bax protein in OvBH-1 cells did not change clearly after PDT. We have previously described a silent mutation in codon 224 in exon 6, found in OvBH-1 cell line [4]. The hypothesis that OvBH-1 cell line does not express wild-type p53 protein, was further confirmed by Western blot analysis and by immunohistochemical staining using Pab1620 anti-p53 antibody [4]. Consequently, we postulate that the lack of apoptosis in OvBH-1 cells may be dependent on the presence of a stable form of p53 protein and the absence of the wild-type p53 protein. The control cell line, MCF-7, containing a wild-type of p53, undergoes apoptosis in response to numerous toxic agents, but it could be mentioned that in PDT-treated MCF-7 cells nuclear-cytoplasmic localization of p53 protein was observed [31]. These results corroborate data of Zhang et al. [32] demonstrating that introduction of wild-type p53 transfected to the p53 null HT29 human colorectal carcinoma cells made them more sensitive to induction of apoptosis after PDT. The MTT assay results revealed that the wild-type p53-expressing transfected cells were approximately two-fold more sensitive to PDT than non-transfected controls [32]. In our study, a restricted direct cytotoxicity of photofrin II-PDT for OvBH-1 cells was observed. We pointed out that the activity of mitochondrial enzymes was measured, but the data may not reflect cell survival .The total absence of apoptosis markers such as Bax protein and the human neoepitope cytokeratin 18 (M30) expression in OvBH-1 cells, which are present on cells ongoing apoptosis, suggests that PDT kill cells by necrosis. Song et al. [26] revealed that hemoporfin-based PDT induced cell death of SKOV3 ovarian carcinoma cell line by necrosis. The authors [26] suggest that type of cell death induced by photocytotoxic effect of hemoporfin-PDT is cell type related. In the present study, the neoepitope cytokeratin 18 (M30) expression accompanied the apoptosis of MCF-7 cells after photofrin II-PDT which is in agreement with the observations of Bantel et al. [3] that the diminution in neoepitope cytokeratin 18 (M30) expression might be the marker of apoptosis. It is worth underlining that, based on the alkaline comet assay, we revealed photofrin II-PDT induced necrosis in OvBH-1 cells and apoptosis in MCF-7 cells. Several data have been published [1, 24] showing that cell death after PDT can occur by necrosis or apoptosis depending on intracellular localization of photosensitizer [24]. A few clinical studies [17, 27] have evaluated the response to platinum-based chemotherapy in patients with clear cells ovarian carcinoma. Patients with clear cell ovarian carcinoma showed a very low response rate, whereas patients with serous ovarian carcinoma had a high response to platinum therapy. We would like to point out that the patient from whom OvBH-1 cell line was derived obtained 4 cycles of cisplatin-based therapy and died 6 months after surgery. Most experimental [5, 8, 26] and clinical [12] studies were performed on serous ovarian carcinoma and showed effective results of PDT not only *in vitro*

but also in clinical studies. A worse clinical outcome and a more aggressive biological behavior of clear cell ovarian carcinoma as compared to other histological types of ovarian cancers [27] indicate that a new treatment strategy for clear cell ovarian carcinoma should be established. In summary, our *in vitro* preliminary investigation indicates that PDT causes non-apoptotic death in OvBH-1 cell line. However, before using PDT in clinical practices further research is needed to fully confirm that PDT might improve therapy of patients with clear cell ovarian cancer.

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