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Proliferating Cell Nuclear Antigen in Neoplastic PC12 and Normal 3T3 Balb Cells After Photodynamic Therapy

Aktywność w komórkach linii nowotworowej PC12 i prawidłowej 3T3 Balb pod wpływem terapii fotodynamicznej

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Abstract

Background. Photodynamic treatment (PDT) is an emerging therapeutic procedure for the management of cancer, based on the used of photosensitizers, compounds that generate highly reactive oxygen species (ROS) on irradiation with visible light.
Objectives. Study of the effect of photodynamic therapy with photosentilizer application (Photofrin II) and laser light on proliferative activity of neoplastic and normal cell lines.
Material and Methods. Effect of photodynamic therapy with photosensitizer application (Photofrin II) and laser light of 632.8 nm wavelength on proliferative activity of neoplastic PC12 and normal 3T3 Balb cell lines was studied. The experiment was conducted at different irradiation times and different times of incubation with photosensitizer. The cells proliferation was analyzed by immunocytochemical method ABC with monoclonal antibodies anti-PCNA.
Results. Decrease in the number of proliferating cells was shown in both neoplastic and normal cell lines. Differences in the proliferating cells number between the two lines were insignificant. In neoplastic line cells PC12 the greatest drop in proliferative activity was found after 1 h and 3 h incubation with photosensitizer at 10-minute irradiation time. In normal line cells 3T3 Balb lowered proliferative activity was noticed after 1 h and 24 h incubation with photosensitizer at 10-minute irradiation, and after 24 h incubation with photosensibilizator at 5-minute irradiation. No significant decrease in proliferating cells number was observed in neoplastic and normal lines without irradiation, mainly after 0 h and 24 h incubation periods. Greater decrease in proliferative activity in PC12 cells was observed after 3 h incubation without irradiation, as compared to 3T3 Balb cells.
Conclusions. The obtained results suggest that photosensitizer (Ph II) has significant influence on proliferative activity of neoplastic and normal cells (**Adv Clin Exp Med 2006, 15, 2, 241–245**).

Key words: photodynamic therapy, PCNA, Photofrin II.

Streszczenie

Wprowadzenie. Terapia fotodynamiczna (PDT) jest nowoczesną i obiecującą metodą wykrywania i zwalczania nowotworów, prowadzącą do rozpadu komórek na drodze fotodynamicznego utleniania. Terapia polega na selektywnym zatrzymywaniu w tkance nowotworowej fotouczulacza, który po aktywacji światłem powoduje powstawanie reaktywnych form tlenu (RFT). Pod wpływem RFT w komórce pojawia się stres oksydacyjny, a w rezultacie następuje śmierć komórki.
Cel pracy. Ocena aktywności proliferacyjnej komórek linii nowotworowej PC12 i prawidłowej 3T3 Balb pod wpływem terapii fotodynamicznej
Materiał i metody. Zbadano wpływ terapii fotodynamicznej z wykorzystaniem fotofrinu II (Ph II), wzbudzanego światłem czerwonym o długości fali $\lambda = 632{,}8$ nm, na aktywność proliferacyjną komórek nowotworowych PC12 i prawidłowych 3T3 Balb. Zastosowano różne czasy naświetlania i różne czasy inkubacji z fotouczulaczem. Aktywność proliferacyjną komórek oceniono metodą immunocytochemiczną ABC z zastosowaniem przeciwciał anty-PCNA.
Wyniki. Dla obu linii komórkowych wykazano zmniejszającą się liczbę proliferujących komórek. Różnice w proliferacji PC12 i 3T3 Balb nie były istotne. Największe zmniejszenie aktywności proliferacyjnej dla PC12 można było zaobserwować po 1 i 3 godzinach inkubacji z Ph II i po 10 minutach naświetlania. Dla linii prawidłowej zmniejszenie aktywności proliferacyjnej stwierdzono dla inkubacji z Ph II po 24 godz. inkubacji i 5 minutach na-

świetlania. Dla obu linii nie zaobserwowano żadnego znaczącego zmniejszenia aktywności bez naświetlania po inkubacji z fotouczulaczem dla 0 i 24 godzin. Największe zmniejszenie wartości indeksu proliferacyjnego obserwowano dla inkubacji po 1 godzinie dla PC12 oraz dla inkubacji po 1 i 3 godzinach dla 3T3 Balb.
Wnioski. Uzyskane wyniki sugerują, że zastosowany fotouczulacz (Ph II) ma znaczący wpływ na aktywność proliferacyjną komórek prawidłowych i nowotworowych (**Adv Clin Exp Med 2006, 15, 2, 241–245**).

Słowa kluczowe: terapia fotodynamiczna, PCNA, fotofrin II.

Photodynamic therapy (PDT) is a therapeutic method directed at neoplastic cells destruction. The factors causing cells disintegration observed during PDT are reactive oxygen species (ROS), emerging during interaction of halogen lamp light or low-power laser light with photosensitizing dye inserted into the cells [1, 2]. Reactions induced in photodynamic therapy are also called photosensitive processes of the first and second types. Type one, the so called free radical mechanism, leads, among others, to superoxide and hydroxyl radicals' formation, during the direct reaction of light excited photosensitizer with neoplastic tissue. This process takes place in conditions of low oxygen concentration in cellular environment [3]. The second type involves singlet oxygen created when photosensitizer in triplet state transmits energy on oxygen molecule. Reactions of singlet oxygen with other molecules result in cellular structures impairment [4, 5]. Access of an appropriate quantity of dye and oxygen into tumour cells (greater than into normal cells) is possible, among others, due to intensive tumour vascularization [6]. The effect of PDT action on blood vessels is their contraction induced by inhibition of nitric oxide formation and release, different cytokine types, as well as the increase of vessel walls permeability and blood flow decrease in neoplastic tissue [7–10].

PDT affects all the intracellular structures. One of the important morphological effects is alteration in cell shape, which unequivocally reflects the therapy factors impact on cytoskeleton proteins [11]. In studies conducted on animal cells DNA fragmentation (both nuclear and mitochondrial) has been shown, which could bring about impairment in genetic information. It is strictly conditioned by physicochemical properties of an applied photosensitizer [10, 12, 13].

Cellular structures disintegration and genetic information modulation induced by PDT diverts neoplastic cells into death pathway [14]. Numerous studies prove that tumour cells exposition on PDT may lead to their death through two separate processes: apoptosis and necrosis [15–18], which is visualized by decrease in proliferative activity of cells.

The aim of the study was the assessment of proliferation activity of neoplastic and normal cells subjected to photodynamic therapy at different incubation time (0, 1, 3 and 24 hours) with photosensitizer Photofrin II (30 μg/ml of Ph II) and different times of irradiation (10 and 15 minutes) with laser light of 632.8 nm wavelength using He-Ne laser (helium-neon laser).

Material and Methods

Cell Lines Culture

The experiments were carried out on two cell lines: normal 3T3 Balb (mouse embrional fibroblast-like cells) and neoplastic cell line PC12 (pheochromocytome cells from rat adrenal gland). Cells were obtained as a gift from the Department of Histology and Embryology.

The cells were harvested in cell culture medium MEM (Sigma-Aldrich) with 3% glutamine, 10% fetal calf serum and antibiotics: gentamycin (100 μg/ml) and penicillin (100 UI/ml). The cell lines were incubated at 37°C with 5% CO_2. Cells were collected from the culture flasks and distributed on the eight-well plate. Subsequently the cells were incubated with 30 μg/ml of photosensitizer – Photofrin II (Ph II) (QLT Phototherapeutics, Inc. Vancouver, Canada).

The cells underwent the following incubation times with Ph II: 0 h, 1 h, 3 h, and 24 h. Irradiation time with laser light of 632.8 nm wavelength was 5 and 10 minutes. The same incubation times with Ph II were applied for non-irradiated cells.

The cells proliferation was analyzed immunocytochemically (ABC method) with the usage of Monoclonal Mouse Anti-Proliferating Cell Nuclear Antigen (DAKO).

Immunocytochemical Studies

Slides with microculture were fixed in buffered formalin for studies with immunoperoxidase method ABC. Endogenous peroxidase was blocked with 1% solution of H_2O_2. After repeated rinsing in PBS, non-specific protein binding was blocked in Protein Block Serum Free (DAKO). The slides were incubated with an antibody against proliferating cell nuclear antigen (PCNA – clone PC10) in commer-

cially available concentration (1 : 50), during 30 min at a room temperature, then rinsed in PBS, and a biotinilated antibody was superimposed (LSAB 2 KIT, DAKO). After PBS rinsing the slides were incubated with streptavidin-peroxidase complex (LSAB 2 KIT, DAKO). Immunocytochemical reaction was triggered with diaminobenzadine (DAB) solution. Then the slides were rinsed with running water and dehydrated in a graded alcohol series. Positive and negative controls were included in each experiment but cut off for PCNA for the sake of low importance for presented analysis. Proportional assessment of proliferative activity of the cells (for a 100 cells in the field of vision) was analyzed in the light microscope Olympus BHS with Nomarski appendage with the lens of 20 xs zoom.

Results

The number of proliferating cells, both in normal 3T3 Balb and neoplastic PC12 lines decreased under the influence of the applied factors of photodynamic therapy. Only minor differences in proliferative index between normal and neoplastic cells after PDT application were observed.

In neoplastic cell line (PC12) the greatest drop in proliferative activity was found after 1 h and 3 h incubation with photosensitizer at 10-minute irradiation time (Fig. 1).

In normal cell line 3T3 Balb lowered proliferative activity was shown after 1 h and 24 h incubation with photosensitizer at 10-minute irradiation time, and after 24 h incubation with photosensibilizator at 5-minute irradiation (Fig. 2).

In non-irradiated cells decrease in proliferating cells both neoplastic and normal was not significant, which was evident mainly after 0 h and 24 h incubation. However, the greatest drop in proliferative activity was observed in PC12 cells after 3 h incubation without irradiation in comparison to 3T3 Balb cells (Fig. 1, 2).

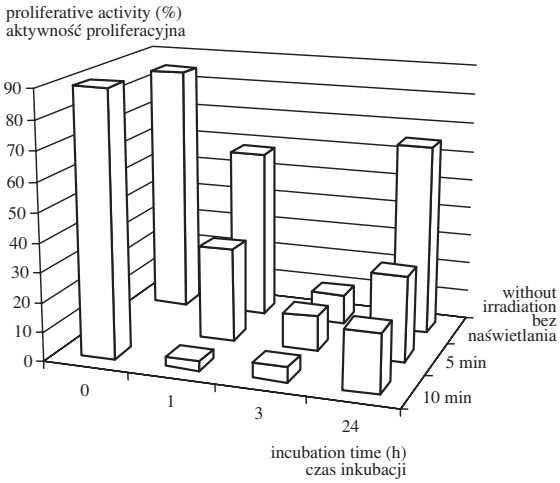

Fig. 1. Proliferative activity of neoplastic cell line (PC12) after photodynamic therapy

Ryc. 1. Aktywność proliferacyjna komórek linii nowotworowej (PC12) pod wpływem terapii fotodynamicznej

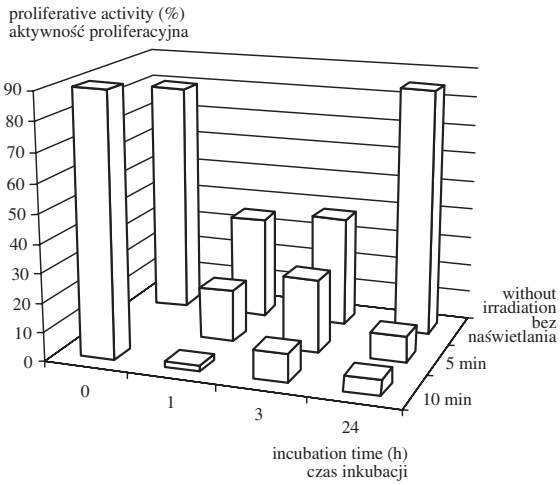

Fig. 2. Proliferative activity of normal cell line (3T3 Balb) after photodynamic therapy

Ryc. 2. Aktywność proliferacyjna komórek linii prawidłowej 3T3 Balb pod wpływem terapii fotodynamicznej

Discussion

The mechanism of photodynamic reaction in tissues is not fully elucidated [5, 19]. The purpose of PDT is selective destruction of neoplastic tissue resulting in decreased proliferative activity of the cells. Many studies have shown that applied in PDT photosensitizers are absorbed better by cancer than normal cells [13, 20]. The results of this study did not show significant differences in proliferative activity drop between normal and neoplastic cell lines, which suggests similar affinity for the applied photosensitizer in both PC12 and 3T3 Balb lines.

The obtained results showed the greatest drop in proliferative index in PC12 cells after 1 h and 3 h incubation with photosensitizer at 10-minute irradiation, and in normal cells after 1 h incubation and the same irradiation time.

An astonishing observation was the increase in proliferative activity in control neoplastic and normal cells after 24 h incubation with photosensitizer without irradiation. It could be assumed that this period allows for removal of the photosensitizer from the cells or its degradation. However, more studies are needed to explain this phenomenon. Photodynamic therapy *in vivo* starts after 24–72 hours, depending on the administration way and a photosensitizer type. It seems that *in vitro* conditions (monolayer) allow for faster photosensitizer

penetration into cells, so the optimal exposition time of cells subjected to PDT *in vitro* is not comparable with *in vivo* conditions.

In normal 3T3 Balb cells decreased proliferative index was shown after 1 h and 24 h incubation with photosensitizer at 10-minute irradiation, and after 24 h incubation with photosensibilizator at 5-minute irradiation (Fig. 2). *In vivo* studies have shown photosensitizers distribution also in healthy tissues, e.g. in liver or skin cells, which indirectly confirms possibility of photosensibilizator (Ph II) accumulation in normal line cells. Uehara and Inokuchi also obtained significantly lower PCNA indicator than in control group in the vascular cell at 24 h after PDT.

Photosensitizer's distribution within an organism is carried out mainly *via* blood system, probably with the help of different protein carriers, but mostly lipid carriers [11, 21]. The carriers' character is conditioned by the used photosensitizer's properties (hydrophilic, hydrophobic, amphiphilic). Hydrophobic dyes show greater efficiency and selectivity of absorption by neoplastic tissue. The involvement of cancer tissue lipoprotein (LDL) receptors in the accumulation of medium and high hydrophobic photodyes has been shown [22]. Complex receptor-LDL-photosensitizer in PDT is absorbed *via* endocytosis. High amounts of photosensibilizator can be inserted into cells with the usage of LDL [13]. Kessel's studies have shown that the pattern of HpD (porphyryn mixture) distribution in lung tumour is correlated with the number of LDL receptors in various tissues [15]. LDL catabolism increases mainly in cells with high proliferative activity, which could also concern healthy cells with high proliferative activity, e.g. fibroblasts. Therefore, it could be assumed that lipoprotein receptors involvement may affect better photosensitizer absorption and transport into cells [13]. Used in this study normal cell line 3T3 Balb is also characterized by high proliferative activity.

So far investigations on PDT have been mainly conducted on neoplastic cell lines; fewer studies have concerned normal cell lines or tumours *in vivo*. In none of the studies by now, proliferative activity under PDT influence has been compared in normal and neoplastic cell lines, with simultaneous application of the same photosensitizer, laser light of the same wavelength and irradiation time. Comparative studies carried out so far have been focused rather on death type analysis (apoptosis, necrosis) of normal and cancerous lines [23].

The results of this study with the application of photosensitizer (Ph II) and laser light (of the same wavelength and irradiation time for both types of cells) did not show significant differences in PDT impact on the cells between normal and neoplastic lines. The obtained results confirm the importance of the comparison between neoplastic cell lines and normal cell lines characterized by low proliferative index.

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