ASSESSMENT OF EARLY APOPTOSIS ON TUMOUR CELL LINE G361 AFTER PHOTODYNAMIC THERAPY

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Abstract

The purpose of this study was to assess whether photodynamic therapy (PDT) induces apoptosis in G361 tumour cell line by using $ZnTPPS_4$ as a sensitiser. A further aim was the construction of an alternative source of radiation, which is suited with its parameters for initiation of the photodynamic phenomenon on carcinoma cells in vitro. At the present time lasers and appropriately high output lamps with filters are most often used in photodynamic therapy. Our prototype source of radiation is planted by light-emitting diodes (LED) emitting at a wavelength range of 455–475 nm. The emitter can be used with the application of a sensitiser showing an absorbing maximum in Soret's strip (e.g. for $ZnTPPS_4$). For the application of other sensitisers absorbing in other regions of the visible electromagnetic radiation spectrum it is necessary to use diodes with appropriate emission characteristics. The optimal density of radiation energy was fixed on 15 $J.cm^{-2}$. About 87 % of dead cells were observed after this treatment following 24 hours of incubation. After 5–15 hours of the incubation we could detect signs of early apoptosis.

Key words

Photodynamic therapy, Flow cytometry, LED, G361

Abbreviations used

PDT,photodynamic therapy; LED, light-emitting diode; DMEM, Dulbecco's Modified Eagle Medium; PBS, phosphate saline buffer; AADK, Annexin V-FITC Apoptosis Detection Kit; PI,propidium iodidee

INTRODUCTION

Photodynamic therapy is a special form of phototherapy. It is a minimally invasive procedure that utilises photosensitising drugs, which are selectively retained by diseased tissues preferentially over normal tissue after their administration to a patient (*1*). The next step of the treatment method is the activation of these drugs

by intense, visible light to achieve selective photochemical destruction of diseased cells and neovasculature (*2*). The basis of the destruction consists in cytotoxic photodynamic reactions using singlet oxygen, which is generated after the sensitiser activation by the appropriate wavelength of light (*3*).

For a maximum efficiency the wavelength of the activation radiation must agree with the absorption peak of the sensitiser (*4*). For PDT, laser systems and classical sources of radiation supplemented with filters may be used. The optimal source depends on the specific application. For example, non-coherent light is not suitable to use in endoscopy due to the big energy loss in light fibres. Therefore, therapists applying PDT rather prefer lasers (*5–8*).

The use of these large, expensive, and inefficient systems is gradually being replaced by the smaller and much cheaper diode systems (*9*). LEDs emit radiation in a range of 350–1100 nm with an energy density of irradiation approximately equalling 150 $mW.cm^{-2}$.

Low price and small size belong to the greatest benefits of using LED sources. Moreover, incoherent radiation can be easily fitted to exposure of a large surface, e.g. in dermic applications (*10, 11*).

The effectivity of PDT can be studied by a flow cytometer. Flow cytometry is a powerful technique for making measurements on single cells. Flow cytometers utilise a sophisticated array of laser, optical devices, and primarily "flow chamber" to measure light scatter and quantify the binding of fluorochrome-labelled antibodies to specific protein antigens on the membrane or inside of cells that are hydrodynamically focused to a single cell wide laminar flow column. The technique also permits to examine living or fixed cells (*12*). We used a flow cytometer to assess the early effect of PDT in a G361 tumour cell line using LEDs as the irradiation source.

MATERIAL AND METHODS

One million human melanoma cells (cell line G361) were displaced to Petri dishes and incubated in DMEM cultivation medium at 37 °C and 5 % CO_2 with 20 μM of $ZnTPPS_4$sensitiser. PDT was induced by a LED source (our product) with a specific radiation wavelength corresponding to the absorption spectrum of the sensitiser used (*13*).

The first part of the Petri dishes was used as a negative control (cells with cultivation medium alone), the second part of the dishes served as a positive control (cells in the presence of 10 μM camptothecin), the third part of the dishes (cells in the presence of 20 μM $ZnTPPS_4$) was irradiated with a dose of 15 $J.cm^{-2}$. After irradiation the cells were cultivated for 5–18 hours at 37 °C and 5 % CO_2.

The cells were subsequently trypsinised (2–5 minutes, at room temperature) and processed by the standard Annexin staining protocol (according to the manufacturer's protocol – performance data sheet) (*14*) and propidium iodide (PI) staining protocol (*15*).

Quantitative measurements of apoptotic and necrotic cells were performed by a flow cytometer (FACSCalibur) using fluorescence probes Annexin V-FITC Apoptosis Detection Kit (AADK, Bio-Vision) and PI (Sigma-Aldrich) in compliance with the standard staining protocol. Positive control dishes with G361 cells were first exposed to 10 μM camptothecin for 4.5 and 13.5 hours. Some dishes were used as a negative control. Other dishes with the addition of 20 μM ZnTPPS4 were irradiated by a LED light source at 37 °C and 5 % CO_2. AADK and PI staining was used to detect the optimal dose of irradiation and to study the type of cell death.

RESULTS

In all experiments we used the same radiation dose of 15 J.cm^{-2}, because our previously obtained results using AADK show that the dose is optimal to induce PDT on a G361 cell line (*13*). Apoptosis analysis of G361 cells untreated (control) or treated with PDT is shown in *Fig. 1A-D*, where region M1 represents apoptotic cells. Twenty-five per cent of apoptotic cells were found in the negative control (cells with cultivation medium alone, *Fig. 1A*). After irradiation we recorded an increase in the number of apoptotic cells. In particular, 32 % or 65 % cells were apoptotic after 5 and 15 hours after irradiation, respectively (*Figs. 1B and 1C*). The

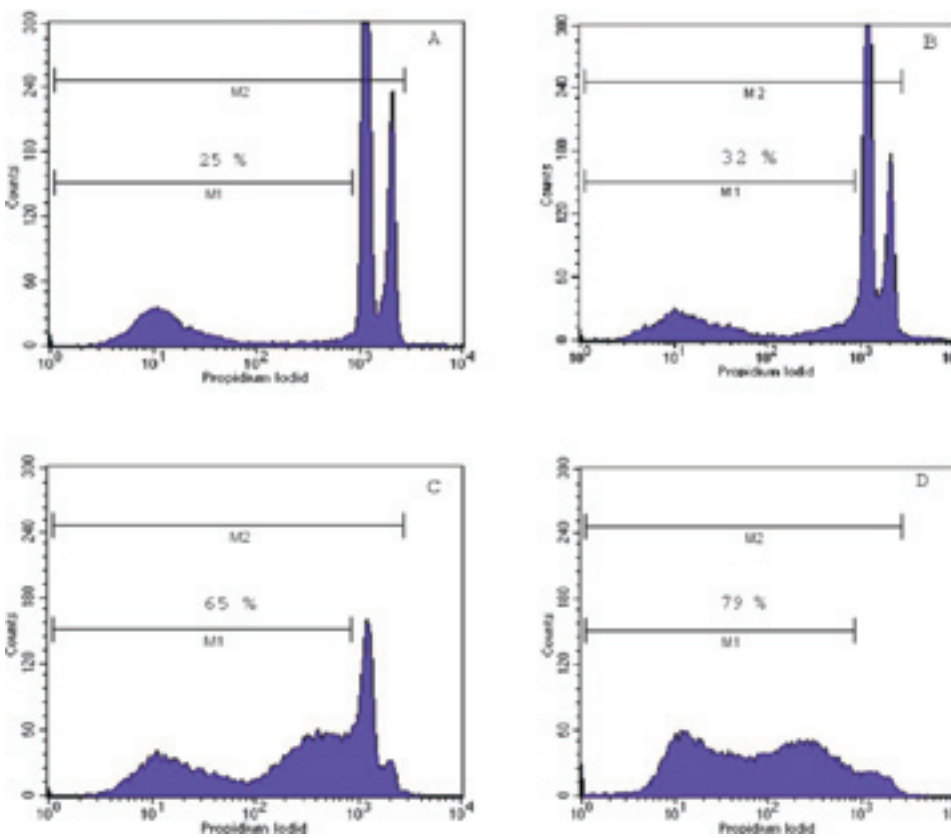

Fig. 1
Cellular DNA content of G361 cells after PDT was quantified by PI staining and flow cytometry. The amount of total apoptotic cells with DNA fragmentation represents M1 peak; M2 peak represents the sum of total cells. (A) Negative control (cells only with medium) (B) Cells after 5 hours after irradiation (cells in the presence of sensitiser), (C) Cells after 15 hours after irradiation (cells in the presence of sensitiser), D) Positive control (cells in the presence of camptothecin)

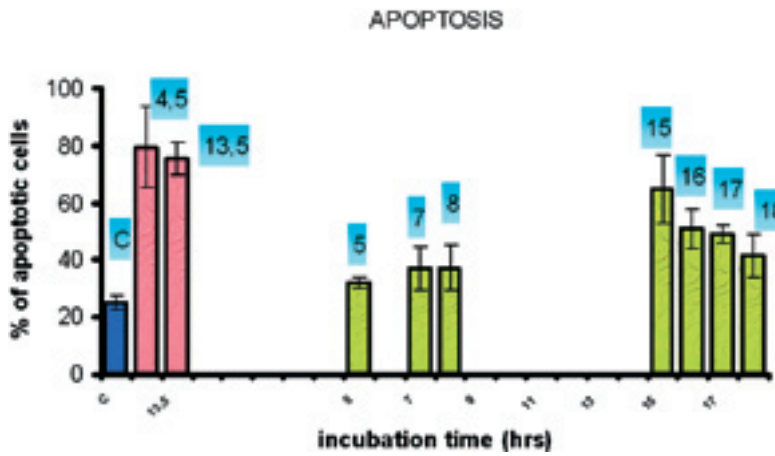

Fig. 2
Progress of apoptosis of melanoma cells after PDT determined by using PI staining and flow cytometry. The 1st peak represents cells with cultivation medium alone – negative control, the 2nd and 3rd represent cells in the presence of 10 μM camptothecin – positive control, bars 4–10 represent cells in the presence of 20 μM $ZnTPPS_4$ after PDT treatment. Incubation time is shown over the bars.

positive control (cells in the presence of camptothecin) shows 79 % of apoptotic cells (*Fig. 1D*). A progress of the apoptosis increase is shown in *Fig. 2*. The first bar represents the negative control, the second and the third bar represent the positive control after 4.5 hours and 13.5 hours incubation, respectively. The rest of the bars (4th–10th) shows the dependence of apoptosis on the incubation time.

DISCUSSION

PDT is characterised by the fact that the radiation dose of visible light used is non-toxic in the absence of sensitiser and the used concentration of sensitiser without light exposure does not exhibit any toxic effect. Radiation doses between 10–25 $J.cm^{-2}$ in combination with $ZnTPPS_4$ as a sensitiser are generally sufficient to induce apoptotic death on a G361 tumour cell line (*16, 17*).

For our study we have chosen the optimal radiation dose of 15 $J.cm^{-2}$. Using PI staining we have found that about 32–65 % of the cells undergo apoptosis after 5–15 hours. Based on the results we can expect that the maximum of early apoptosis will be between 8–15 hours after PDT treatment.

Several groups have reported that PDT can induce cell death with a variety of photosensitising agents, including porphycene derivatives, chloroaluminum sulfonated phthalocyanine, Photofrin II, and 5-amino-levulinic acid. However, the relative contributions of apoptosis and necrosis are dependent on the cell line, photosensitising agent, and/or experimental conditions including methods of

evaluation (*18, 19, 20*). For example, *Chen at al.* found out that PDT using motexafin lutetium induces up to 35 % increase in the apoptosis of vascular cells (*18*).

A prototype light source (LED) was found to be a practical device (*21*). LEDs are compact, lightweight, and require significantly less energy to produce the desired wavelengths of light applicable in photodynamic therapy (*22*). Photodynamic therapy on a G361 cell line induced by a LED source has now been demonstrated to be feasible, safe, and effective to provoke early apoptosis.

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STANOVENÍ ČASNÉ APOPTÓZY U NÁDOROVÉ LINIE G361 PO FOTODYNAMICKÉ TERAPII

Souhrn

Účelem této studie bylo zjistit, zda při použití viditelného světla a $ZnTPPS_4$ jako senzitizéru dojde k indukci apoptózy v nádorové buněčné linii G361. Dalším cílem bylo sestrojení alternativního zdroje pro ozařování, který bude svými parametry vyhovovat pro navození fotodynamického jevu in vitro. Námi sestrojený ozařovač je osazen LED diodami zářícími v rozmezí vlnových délek 455–475 nm. Lze jej tedy použít v kombinaci se senzitizéry, které mají absorpční maximum v Soretově pásu, což je například senzitizér $ZnTPPS_4$. V případě aplikace senzitizéru s jiným absorpčním spektrem je nutné použít odlišné diody s vhodnou vlnovou délkou. Optimální hustota záření (dávka) byla stanovena na 15 $J.cm^{-2}$, kdy bylo pozorováno po 24hodinové inkubaci 87 % buněk mrtvých. Po aplikaci této dávky jsme pozorovali maximum časné apoptózy mezi 5–15 hodinami po ozáření..

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