Effects of Photodynamic Exposure on Endothelial Cells *In Vitro*

E. R. Andreeva, O. O. Udartseva, I. N. Vozovikov, S. G. Kuzmin, and E. M. Tararak

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Experiments on cultured human umbilical vein endotheliocytes showed that accumulation of photosensitive dye (aluminum phthalocyanin; PHOTOSENSE) in cells and laser exposure alone were inessential for the viability of endothelial cells. Contrary to this, exposure of the cells which have accumulated aluminum phthalocyanin (an average of 111.1 ng/mg protein) to low-intensity laser (λ=675 nm) led to a dose-dependent reduction of endotheliocyte viability. Hence, cultured endothelial cells can be used for screening of various photosensitizers and preliminary optimization of photodynamic therapy.

Key Words: *arterial proliferative diseases; umbilical vein endothelium; cell culture; photodynamic exposure*

Photodynamic exposure or photodynamic therapy (PDT) is a method consisting in systemic injection of an inert substance (photosensitizer) and subsequent local exposure of cells with accumulated dye to low-intensity laser. This results in the formation of reactive oxygen species producing a cytotoxic effect [4]. PDT is now widely used in clinical oncology [4,9] as a method for noninvasive selective modification of tumor cells. The possibility of using PDT in other branches of medicine is now investigated; for example, for prevention and therapy of proliferative diseases of arteries associated with the development of intimal hyperplasia, e.g. atherosclerosis, postangioplastic restenosis, *etc*. [6,10,11,13]. Specific features of photodynamic exposure of various cell types (endothelial, smooth muscle cells, monocytes/macrophages, lymphocytes) in the vascular wall are essential for effective development of approaches to PDT of arterial diseases. The first object of the exposure in intravascular PDT will be the endothelial monolayer cells, lining the inner surface of arteries. Hence, study of endotheliocyte sensitivity to photodynamic exposure is an obligatory preliminary stage in the creation of a PDT protocol for the treatment and prevention of proliferative arterial diseases. These studies are carried out on cells isolated from human body and cultured *in vitro*.

We measured accumulation of aluminum phthalocyanin (photosensitive dye; PHOTOSENSE, PS) in cultured endotheliocytes and studied the effects of PS accumulation, low-intensity laser exposure, and photodynamic exposure (laser exposure of cells which accumulated PS) on the viability of endotheliocytes.

MATERIALS AND METHODS

Primary cultures of endotheliocytes from human umbilical vein were obtained as described previously [1]. The cells were cultured in medium 199 (Biolot) with 10% FCS (PAA), 100 μg/ml heparin (Moscow Endocrine Plant), 200 μg/ml endothelial cell growth factor (ECGF), 2 mM L-glutamine, 50 U/ml penicillin, 50 μg/ml streptomycin, 25 μg/ml fungizone (all reagents from Sigma). Accumulation of PS and the effects of photodynamic exposure on cells were studied on endotheliocytes of passages 2-4.

Institute of Experimental Cardiology, Russian Cardiology Research-and-Production Complex, Federal Agency for High-Technology Medical Care, Moscow, Russia. ***Address for correspondence:*** andreeva-er@mail.ru. E. R. Andreeva

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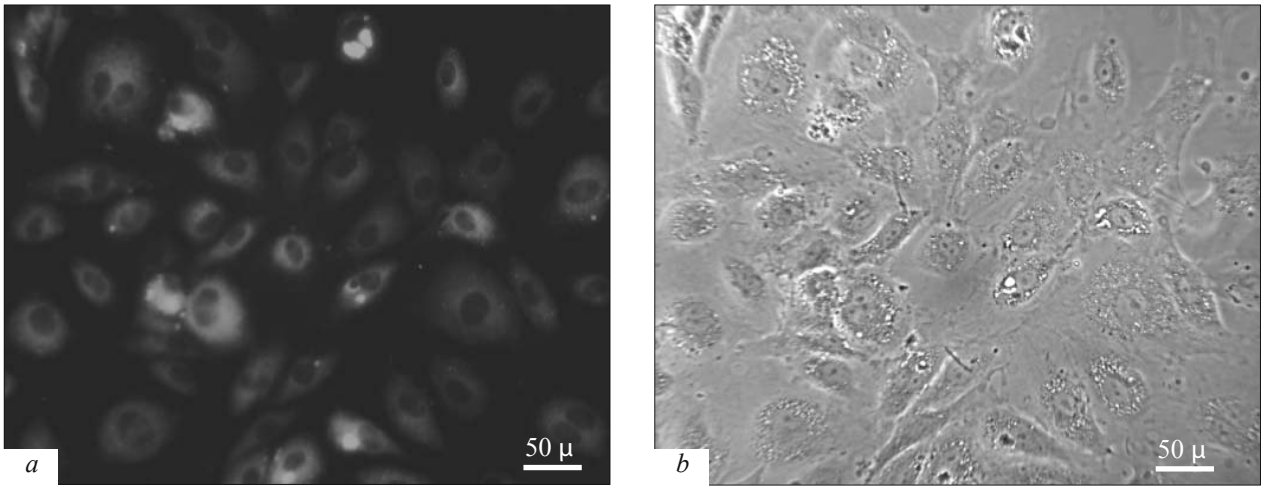

Fig. 1. Cultured human umbilical vein endotheliocytes. Passage 2. *a*) after 24-h incubation with PS virtually all cells containing the dye. Fluorescence (λ_{ex}=340-380, λ_{em}=425); *b*) the same field of view, phase contrast.

The PS in a concentration of 10 μg/ml blood is used in PDT for cancer; the photodynamic exposure is carried out 24 h after injection of the agent. We used the same PS concentration in culture medium and the same interval in our *in vitro* experiments.

The endothelial cells were cultured unitl confluence; 24 h before the experiments, the PS was added to the culture medium. The PS was a mixture of di-, tri-, and tetrasulfonated aluminum phthalocyanin salts (Institute of Organic Semiproducts and Stains) in a concentration of 10 μg/ml. The culture medium was replaced with fresh one (without PS) directly before laser exposure.

PS fluorescence was visualized in a Leica DM 5000B fluorescent microscope (Leica GmbH) fitted with an HBO 100AC mercuric lamp, a set of UV filters (G 365, LP 420), and computer system for image recording.

Accumulation of PS in endothelial cells was measured spectrofluorometrically as described previously [3]. Endotheliocytes were incubated (24 h) with PS, after which the cells were washed and harvested with 0.05% trypsin and 0.04% EDTA. The suspension was centrifuged and the cell precipitate was dissolved in 0.1 M NaOH at 60°C for 1 h. PS fluorescence intensity was measured in a half of the cell lysate on a Schimadzu spectrofluorometer (λ_{ex}=608 nm, λ_{em}=687 nm). The concentration of PS in the lysate was determined using a calibration curve plotted for the fluorescence intensity of samples with known concentrations of PS. Protein concentration in the lysate was measured by the method of Lowry. PS concentration in endotheliocytes was standardized per mg cell protein.

TABLE 1. Accumulation of PS in Human Umbilical Vein Endotheliocytes

Experiment No.	Content of PS, ng/mg protein
1	153.2±12.6
2	120.1±4.1
3	78.5±4.1
4	92.7±36.5

PS-loaded cells and cells without dye were exposed on an Azor-PDT 675 laser device for PDT (Azor company) at λ=675 nm at irradiation doses of 0.5 to 100 J/cm^2.

Cell viability was measured by colorimetry using MTT test.

Statistical analysis was carried out using Microsoft Excel 2000 software. The arithmetic mean and error of the mean were calculated.

RESULTS

It is known that phthalocyanins upon stimulation with UV light fluoresce in the red band, due to which the dye in cells is visualized. After 24-h incubation, diffuse accumulation of PS mainly in the perinuclear area of endotheliocyte cytoplasm was seen (Fig. 1). In some cases, granular structures containing PS were detected. Virtually all endothelial cells contained the dye after 24-h incubation with PS.

Endothelial cell capacity to accumulate PS varied from one experiment to another, presumably because of individual characteristics of cells from different donors (each experiment was carried out on EC isolated from the umbilical vein of one donor; Table 1). The mean content of PS per cell was 111.1 ng/mg protein (data of 4 experiments).

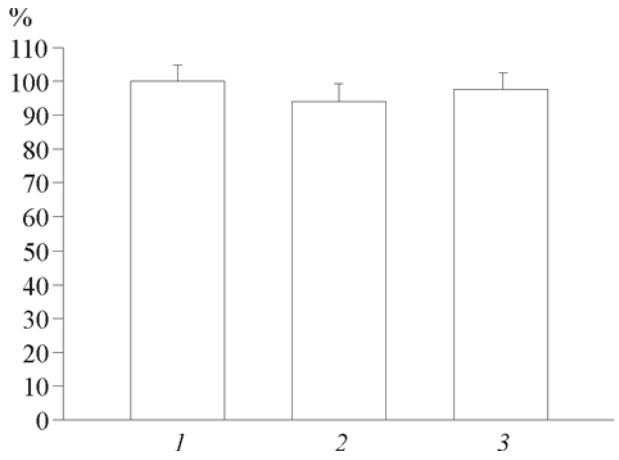

Fig. 2. Effects of PS accumulation and laser exposure on viability of human endothelial cells. *1*) intact endothelial cells (control); *2*) endotheliocytes loaded with PS; *3*) endotheliocytes exposed to 2 J/cm^2. Here and in Fig. 3: ordinate: % of viable *vs*. intact cells. Confidence intervals at $p<0.05$. Results of a representative experiment ($n=6$) are shown.

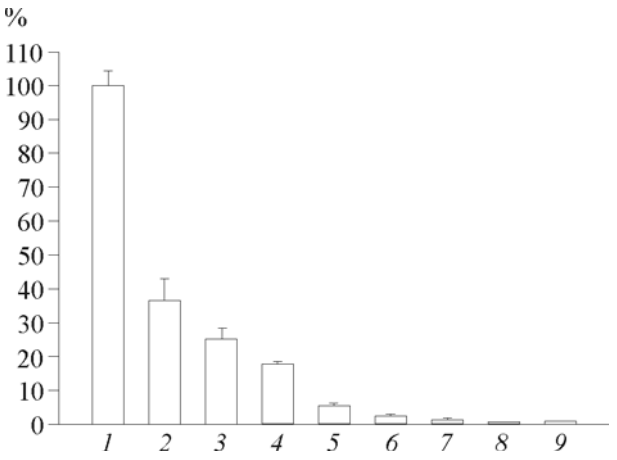

Fig. 3. Effect of photodynamic exposure on viability of human endotheliocytes. *1*) intact endothelial cells (control); *2-9*) photodynamic exposure (endotheliocytes loaded with PS exposed to laser (λ=675 nm) in different doses: *2*) 0.5 J/cm^2; *3*) 1 J/cm^2; *4*) 2 J/cm^2; *5*) 5 J/cm^2; *6*) 10 J/cm^2; *7*) 20 J/cm^2; *8*) 50 J/cm^2; *9*) 100 J/cm^2.

Analysis of the counts of viable endotheliocytes after 24-h incubation with PS and after laser exposure showed that none of these factors alone damaged the cells (Fig. 2). On the contrary, viability of endothelial cell after photodynamic treatment (laser exposure of endotheliocytes containing PS) decreased with increasing the exposure dose from 0.5 to 100 J/cm^2 (Fig. 3). The LD_{50} for endothelial cells in 6 independent experiments was 0.5-1 J/cm^2.

The majority of authors think that human cultured endothelial cells are a convenient test system for studies of various aspects of photodynamic exposure of the cells [2,5,7,8,12,14]. It was shown, for example, that photodynamic exposure of cultured endothelial cells caused partial shrinkage of cells and stimulated (in a dose-dependent manner) neutrophil adhesion to extracellular matrix proteins, exposed as a result of this shrinkage [7]. It was also found that the thrombogenic status of endotheliocytes changed after PDT because of reduced expression of thrombomodulin by these cells and by increased production of tissue factor [8]. Comparison of the sensitivity of cultured capillary endotheliocytes to photodynamic exposure with 5-aminolevulinic acid and photofrine as photosensitizers [5] demonstrated higher cytotoxicity of the latter agent. In our studies, PS accumulated in cultured endothelial cells within 24 h exhibited no cytotoxic effect. In addition, laser exposure *per se* did not reduce viability of cultured cells. Combined use of PS and laser exposure dose-dependently reduced the number of viable endothelial cells.

Hence, *in vitro* use of endothelial cells is a convenient system for evaluating cell sensitivity to various photodynamic agents (photosensitizers and laser), comparing the efficiencies of the known or new photosensitizers, evaluating the parameters of laser exposure, visualizing the photodynamic effects manifesting by the formation of active oxygen species, and studying the impact of photodynamic exposure options for the mechanisms of cell death in PDT.

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