Low-Fluence Photodynamic Treatment Modifies Functional Properties of Vascular Cell Wall

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We studied the effect of low-fluence photodynamic treatment with Photosence preparation on functional properties of macrophages and endothelium *in vitro*. It was shown that low-intensity photodynamic treatment did not affect viability of these cells. Exposure (0.25 J/cm^2) of endothelial cells loaded with Photosence did not change the expression of adhesion molecules, but reduced adhesion of peripheral blood mononuclears to these cells. The same low-intensity exposure inhibited phagocytic activity of macrophages and reduced activity of matrix metalloproteinase-9 produced by them.

Key Words: *photodynamic treatment; vascular wall cells; atherosclerosis*

Photodynamic treatment (PDT) is a two-component non-invasive method consisting of systemic administration of a non-toxic photosensitizer (PS) followed by local low-intensity laser irradiation [5]. Photochemical transformation of the dye induced by light of a certain wavelength yields reactive oxygen species toxic for the cells. Since the cells considerably differ by the capacity to accumulate PS [1,15], this method allows selective elimination of specific cell populations.

PDT is now widely used in clinical practice for the treatment of tumor diseases. Some authors proposed to use PDT for the treatment of proliferative arterial diseases, *e.g.* atherosclerosis [3,8,12]. Atherosclerotic injury is a result of complex interaction of several cell types; therefore, the target cells for PDT cannot be definitely specified. Endothelial cells (EC) and macrophages (MP) playing a crucial role in atherogenesis are of special importance in this respect. Endothelial dysfunction is an early sign of atherosclerosis; it is accompanied by adhesion of peripheral blood mononuclears to the endothelium and their subsequent migration to the subendothelial space, where monocytes differentiate into MP [7]. MP located in atherosclerotic focuses accumulate lipids and secrete bioactive substances, *e.g.* proinflammatory cytokines promoting the injury, and metalloproteinases loosening the structure of atherosclerotic plaques. Regulation of the content of MP and functional activity of all cells of the vascular wall can be an effective approach to reducing the severity of atherosclerotic changes. Our previous *in vitro* experiments showed that the count of cells in the vascular wall can be controlled by PDT. EC are more susceptible and MP most resistant to PDT [1,15], *i.e.* high-dose irradiation of the plaque during PDT will most probably lead to endothelial death, exposure of the subendothelial prothrombogenic surface, and thrombosis, which is extremely undesirable. In contrast to tumors, complete elimination of all cells in not the aim of therapy in case of atherosclerotic injury. Arrest of atherosclerosis progress achieved via modulation of functional activity of cells involved in the formation of plaques is a good positive effect of therapy. PDT with low-dose irradiation can be applied in this case.

Here we studied the effect of low-fluence PDT on functional properties of EC and MP *in vitro*.

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MATERIALS AND METHODS

Monocytes were isolated from human peripheral blood by centrifugation in Ficoll-Hystopaque (Sigma) density gradient and cultured according to a standard protocol in DMEM growth medium (BioLot) supplemented with 10% FCS (HyClone), 2 mM L-glutamine (PanEko), 100 U/ml Penicillin, and 100 μg/ml streptomycin (PanEko). The culture of adherent cells grown for 7 days was considered as primary MP culture [4] and used in the experiments.

Primary culture of EC from human umbilical cord vein was isolated as described previously [2]. The cells were cultured by standard protocols in medium 199 (EC; BioLot) supplemented with 10% FCS, 100 μg/ml heparin, and 200 μg/ml EC growth factor.

The cells were incubated with PS Photosens (10 μg/ml) for 24 h, thoroughly washed from the dye, and irradiated with a diode laser (AZOR PDT, λ=675 nm, doses 0.25-1.00 J/cm^2). The effects of PDT on cell viability, phagocytic activity, and activity of matrix metalloproteinase-9 (MMP-9) produced by MP were evaluated 24 h after irradiation. Cell viability was determined using MTT test. The expression and proteolytic activity of MPP-9 in cell lysates were analyzed using the method of gelatin zymography [17]. Quantitative evaluation of the obtained zymograms was performed using Image J software (NIH).

For measuring phagocytic activity, latex particles (Polyscience) were added to MP in a concentration of 2×10^7 particles/ml for 2 h. Then the cells were washed 2 times with PBS to remove all particles from the cell surface and fixed with methanol (-20°C). Phagocytic index was calculated as the percent of phagocytic MP from the total number of cells.

The effect of PDT on the interaction of endothelium and peripheral blood mononuclears was studied on EC preliminary loaded with PS and irradiated in a dose of 0.25 J/cm^2. One hour after this treatment, suspension of mononuclears preliminary stained with DiO was added (on 30 min). Then, nonadherent leukocytes were removed and the cells were washed with serum-free medium and analyzed by the method of fluorescent microscopy. The data were presented as the mean number of adherent mononuclears per 1000 EC.

Intergroup differences were analyzed using nonparametric Mann–Whitney *U* test.

RESULTS

The cytotoxic effect of PDT depended on the dose of irradiation and cell type (Fig. 1). EC are more susceptible to PDT than MP, which agrees with previous findings [1,15]. It was demonstrated that the dose >0.5 J/cm^2 produced a pronounced damaging effect on the endothelium. Therefore, the doses of 0.25 and 0.5 J/cm^2 were chosen for further experiments.

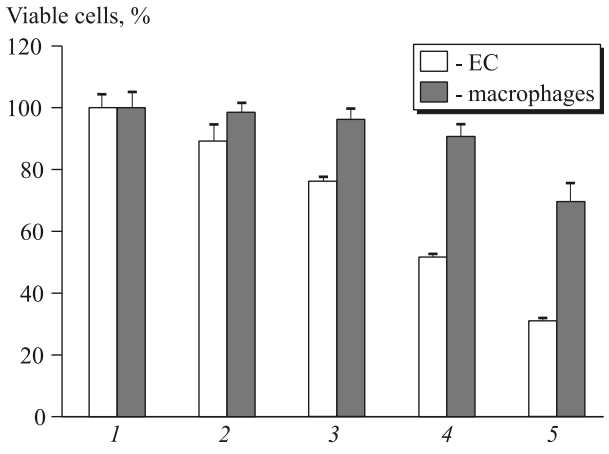

Fig. 1. Cytotoxicity of PDT with administration of Photosence followed by irradiation in different doses. *1*) without irradiation, *2*) PDT, 0.25 J/cm^2, *3*) PDT, 0.5 J/cm^2, *4*) PDT, 1 J/cm^2, *5*) PDT, 2 J/cm^2.

At the next stage, the effect of low-fluence PDT on functional properties of cells was analyzed.

In experiments for evaluation of the interaction of peripheral blood mononuclears with the endothelium, we used preliminary activated with TNF-α and non-activated EC after PDT. It was also shown that low-fluence PDT reduced adhesion of mononuclears to activated EC (Fig. 2) and practically does not affect the interaction of mononuclears with non-activated endothelium (Fig. 2, *c*). Changes in leukocyte adhesion to EC usually correlate with changes in the expression of endothelial adhesion molecules [10]. According to our findings, low-fluence PDT reduced the expression of VCAM-1 and E-selectin by EC activated with TNF-α (Fig. 3). No changes in the expression of ICAM-1 and PECAM-1 were noted.

Apart from EC, MP play an important role in the development of atherosclerosis. Their main activity in the zone of injury is related to phagocytosis mediating lipid accumulation and with production and release of active substances into the surrounding tissue. We also studied the effect of low doses on phagocytic activity of MP and activity of MPP-9 synthesized by them. We found that irradiation of PH-loaded MP in doses of 0.25 and 0.5 J/cm^2 reduced their phagocytic index by 1.5 and 2.4 times, respectively (Fig. 4). Activity of MPP was assayed by the method of gelatin zymography. Expression of MMP-2 was practically not detected in samples; therefore, we studied the effect of PDT on MPP-9 activity. It was found that low-fluence PDT reduced the expression and proteolytic activity of MPP-9 and its precursor pro-MPP-9 (Fig. 5).

Thus, our findings suggest that low-fluence PDT considerably modifies functional properties of vascular wall cells without producing damaging effect.

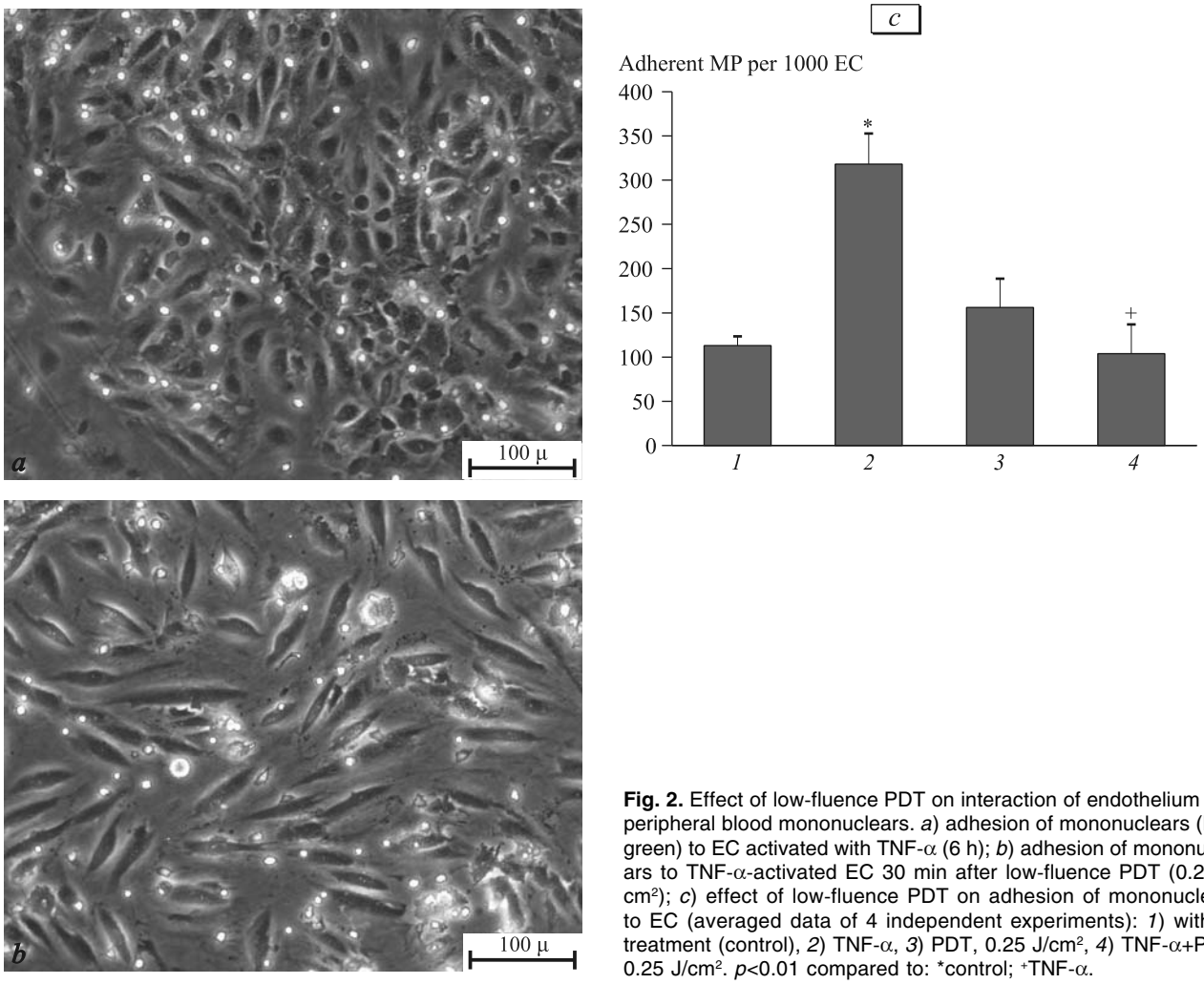

Fig. 2. Effect of low-fluence PDT on interaction of endothelium and peripheral blood mononuclears. *a*) adhesion of mononuclears (MN, green) to EC activated with TNF-α (6 h); *b*) adhesion of mononuclears to TNF-α-activated EC 30 min after low-fluence PDT (0.25 J/cm^2); *c*) effect of low-fluence PDT on adhesion of mononuclears to EC (averaged data of 4 independent experiments): *1*) without treatment (control), *2*) TNF-α, *3*) PDT, 0.25 J/cm^2, *4*) TNF-α+PDT, 0.25 J/cm^2. $p<0.01$ compared to: *control; $^+$TNF-α.

We found only few reports on the effects of low-fluence PDT and their mechanisms [14,16]. Widely used method of PDT with high-dose irradiation is aimed at elimination of defective cells (*e.g.* tumor cells). Under these conditions, the inflammatory process induced by massive cell death produces a favorable effect, because it increases immunogenicity of tumor cells and promotes their destruction by immune system cells [6]. When using PDT for the treatment and prevention of atherosclerotic changes, specific regulations of their formation should be taken into account. Atherosclerotic lesion is a focus of chronic inflammation in the vascular wall [10]; therefore, the development of acute inflammation focus during PDT is extremely undesirable, because it leads to thrombosis and other unfavorable consequences. In this case, low-fluence PDT should produce an anti-inflammatory effect, *i.e.* suppress proinflammatory activity of EC and MP.

The interaction of activated endothelium with peripheral blood leukocytes is an initial stage of vascular wall injury and plays an important role in atherosclerosis development [10]. Monocytes migrating into the subendothelial space are transformed into macrophages playing a role in the formation of inflammation focus. We found that low-fluence PDT reduced adhesion of mononuclears to EC. The number of flattened leukocytes also considerably decreased, which attests to deceleration of their activation. These findings suggest that low-fluence PDT suppresses proinflammatory activity of EC.

The interaction of leukocytes with EC and their subsequent migration are regulated by specific adhesion molecules (ICAM-1, VCAM-1, PECAM-1, E-selectin, and VE-cadherin); expression of these molecules is changed in EC dysfunction [10,13]. According to our findings, low-fluence PDT insignificantly reduced the expression of VCAM-1 and E-selectin and did not change the expression of ICAM-1 and PECAM-1. It can be hypothesized that suppression of mononuclear adhesion to EC is determined by changes in endothelium permeability, which depends on distribution of VE cadherin in the zone of cell-cell contacts [9].

Endothelial dysfunction is associated with enhanced migration of peripheral blood mononuclears to

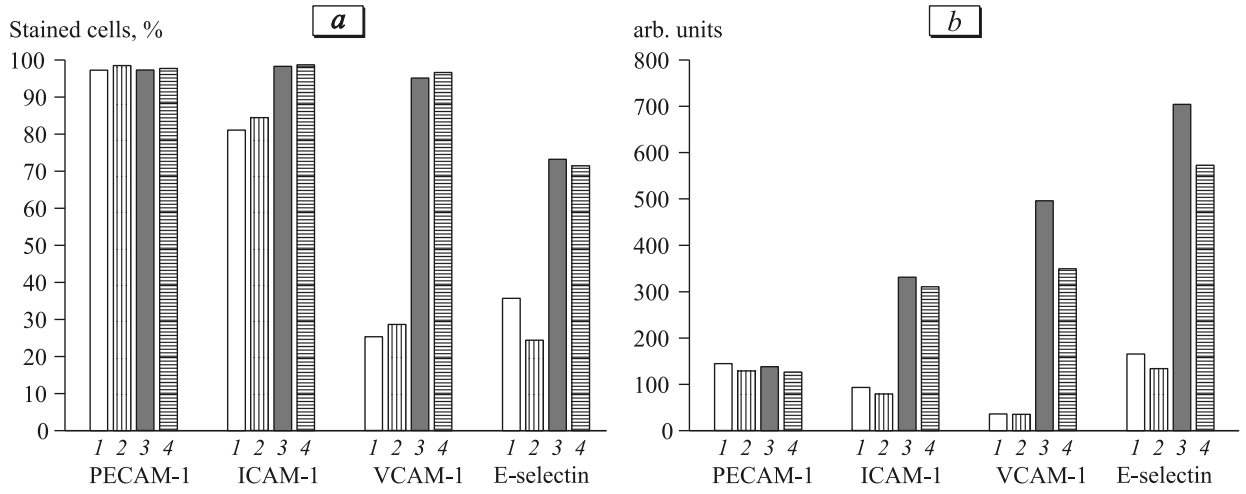

Fig. 3. Effect of low-fluence PDT on expression of adhesion molecules by EC; flow cytofluorometry data. *a*) percent of cells expressing PECAM-1, ICAM-1, VCAM-1, and E-selectin; *b*) mean fluorescence intensity per cell (reflects expression of adhesion molecules by EC). Data of a representative experiment (n=3). *1*) without treatment; *2*) PDT, 0.25 J/cm^2, *3*) TNF-α, *4*) TNF-α+PDT, 0.25 J/cm^2.

the subendothelial space. In the intima, monocytes phagocytize modified low-density lipoproteins and is transformed into MP and then (after excessive accumulation of lipoproteins) into foamy cells. Various factors produced by activated macrophages and foamy cells promote progression of atherosclerotic changes by stimulating additional migration of mononuclears and development of local inflammation in the vascular wall [10]. Excessive accumulation of lipoproteins is primarily determined by phagocytic capacity of cells. In our experiments, low-dose irradiation of MP loaded with PS leads to suppression of their phagocytic activity.

Apart from enhanced phagocytosis, proinflammatory activity of MP in the lesion manifests in secretion of cytokines, chemokines, and proteolytic enzymes [13]. Enhanced expression and high activity of MPP leads to degradation of extracellular matrix and ulceration of the atherosclerotic plaque [11]. Our experiments showed that low-fluence PDT reduced proteolytic activity of MPP-9 and pro-MPP-9 synthesized by MP. Therefore, it can be assumed that low-fluence PDT can stabilize plaques liable to rupture.

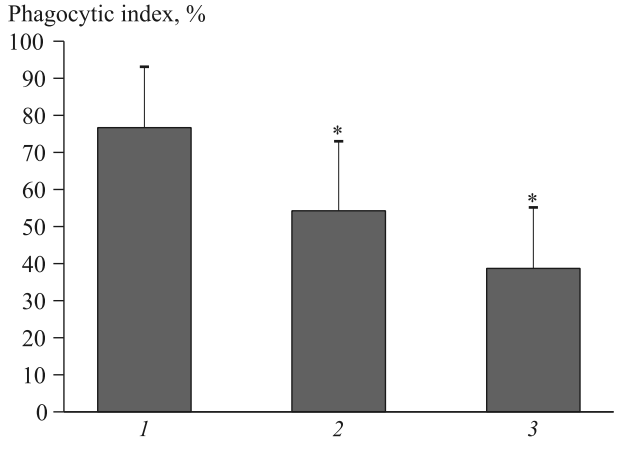

Fig. 4. Phagocytic activity of MP before and after low-fluence PDT. Results of 4 independent experiments are presented. *1*) without treatment (control), *2*) PDT, 0.25 J/cm^2, *3*) PDT, 0.5 J/cm^2. *p<0.01 compared to the control.

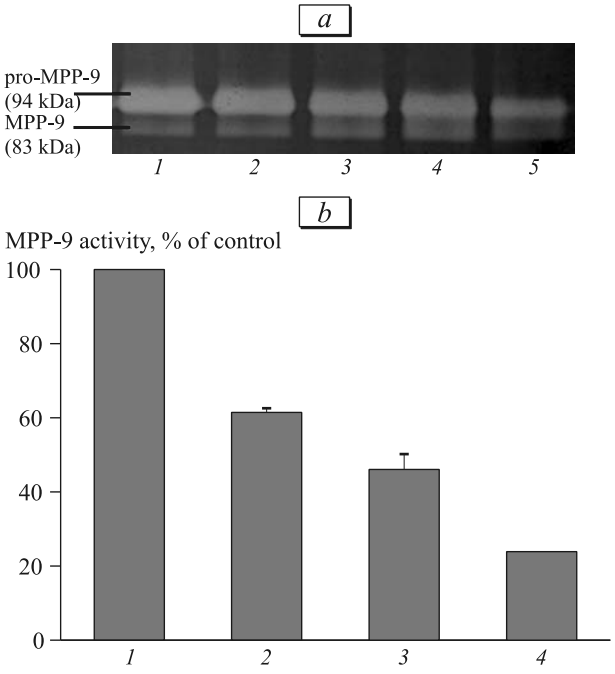

Fig. 5. Effect of low-fluence PDT on proteolytic activity of pro-MPP-9 and MPP-9. *a*: zymography of MP lysates before and after low-fluence PDT: *1*) control, *2*) PS, *3*) PDT, 0.25 J/cm^2, *4*) PDT, 0.5 J/cm^2; *b*: averaged data of densitometric analysis of zymograms (n=3). *1*) without treatment, *2*) PDT, 0.25 J/cm^2, *3*) PDT, 0.5 J/cm^2, *4*) PDT, 1 J/cm^2.

Thus, low-fluence PDT can produce a pronounced anti-inflammatory effect manifested in reduced adhesion of mononuclears to EC and suppression of phagocytic activity of MP. These findings suggest that low-fluence PDT can be an effective method reducing manifestations of atherosclerotic alterations, because this approach reduces proinflammatory activity of EC and MP playing the key role in the development of atherosclerotic lesion [1,13].

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