

# Influence of Low-power Laser Illumination on Pro-inflammatory Response in Human Endothelial Cells Stimulated with Interleukin-1 $\alpha$

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**Abstract.** *Background:* Regarding recent findings on the proven influence of laser light on healing and endothelial regeneration processes, this study was conducted in order to examine the influence of low-power laser illumination on the endothelial inflammatory response. *Materials and Methods:* Human umbilical vein endothelial cells (HUVECs) isolated from umbilical cord, cultured in standard conditions, were harvested and passaged in 24-well plates. Laser influence on HUVEC inflammatory response was measured by stimulating them with Interleukin-1 $\beta$  (IL-1 $\beta$ ) followed by laser light illumination. A 808-nm wave length laser diode and light energy doses of 1.5 and 4.5 J/cm<sup>2</sup> were used (50 mW for 90 and 270 s respectively). The response was measured by assessing Cluster of differentiation 54 (CD54), Cluster of differentiation 62E (CD62E), Monocyte chemoattractant protein-1 (MCP-1) expression and von Willebrand factor (vWF) release, at 6 and 24 h after stimulation. The MCP-1 and vWF activity in cell supernatants was measured with an enzyme-linked immunosorbent assay (ELISA) kit. Cytofluorometry was used to assess CD54 and CD62E expression. *Results:* MCP-1 concentration in supernatants from HUVECs was significantly lower 6 h after 4.5 J/cm<sup>2</sup> stimulation compared to IL-1 $\beta$ -stimulated cells. No changes in MCP-1 levels after IL-1 $\beta$  stimulation plus 1.5 J/cm<sup>2</sup> illumination, compared to IL-1 $\beta$  stimulated HUVECs were noted. IL-1 $\beta$  stimulation significantly enhanced the concentration of vWF and the expression of CD54 and CD62E. Both energies of laser light illumination inhibited the IL-1 $\beta$ -induced increase of CD54 and CD62E concentration. vWF activity after illumination was comparable to that of unstimulated cells. There were no significant differences in the viable cell count between the

groups tested. *Conclusion:* Low-power laser illumination diminishes the pro-inflammatory and pro-coagulant activity of IL-1 $\beta$ -stimulated HUVECs.

Although it has been shown that low-power laser illumination may stimulate bone healing (1-3), endothelial regeneration (4), smooth muscle relaxation (5) and reduce restenosis rate after coronary angioplasty procedures (6-9), little is still known about cellular mechanisms underlying these effects. Laser light is believed to influence the inflammatory process, a major factor during early events in the restenosis cascade (10-12).

CD54 known also as intercellular adhesion molecule-1 (ICAM-1) is an adhesion molecule crucial in inflammatory process by controlling monocyte endothelium interactions. It plays an important role in vasculitis and monocyte migration towards inflamed tissues (13). The importance of CD54 in the restenosis cascade was shown by Kamijikkoku *et al.* who found elevated levels of soluble CD54 in patients with early restenosis (14). Many other publications confirm the importance of CD54 in neointima formation (15-17). Kollum *et al.* reported that systemic application of anti-CD54 monoclonal antibodies diminished restenosis in rabbits (18). The previous studies suggest that the potential mechanism of neointimal growth diminution is inhibition of monocytes transmigration through the regenerating endothelium (19) but the observations of Yakusawa *et al.* open the intriguing possibility that other, unknown CD54-dependent mechanisms play a role in restenosis. They found that anti-CD54 antibodies reduce neointima formation without influencing the number of monocytes/macrophages in the vessel walls (20). It was postulated that cell proliferation may also be enhanced by fibrinogen binding to CD54 (21).

Monocyte chemoattractant protein-1 (MCP-1) is the most important chemotactant protein contributing to the accumulation of mononuclear leucocytes after vessel injury (22). In addition to promoting the migration of monocytes MCP-1 exerts other pro-inflammatory effects including

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induction of monocytes superoxide anion (23) and cytokine production (24). Chemotaxis and recruitment of monocytes have been considered to be critical events of restenosis in humans (10, 11). In this scenario monocytes adhere to the site of injury, accumulate in the vessel wall and release mediators of inflammation which stimulate activation, migration and proliferation of smooth muscle cells resulting in intimal hyperplasia. MCP-1 was shown to be an independent predictor of late loss after coronary angioplasty in humans. There are reports suggesting that accumulation of monocytes after angioplasty led to enhanced luminal re-narrowing (12). In recently published articles new strategies of local and systemic inhibition of MCP-1 action reduced neointima formation in animal models (25-27). The importance of MCP-1 in neointimal growth was previously illustrated by Stark *et al.* who showed that up-regulation of *MCP-1* gene in vein grafts lead to obliterative stenosis and graft failure (28). Moreover Guzman *et al.* reported inhibition of neointima formation with antibodies against MCP-1. (29) Furukawa *et al.* reported prevention of intimal hyperplasia by neutralization of MCP-1 before and immediately after arterial injury (30).

Both CD54 and MCP-1 are critical in the generation of the inflammatory or immune response (31-34) which in the same time are the most important steps in the early restenosis cascade.

VWF, produced mainly by endothelium and megacariocytes, contributes to platelet aggregation and plays a key role in the adhesion of platelets to vascular wall. VWF also protects clotting factor VIII from rapid degradation, which is crucial for sufficient blood coagulation (35). It is known that post angioplasty platelet adhesion and activation accompanied by the release of many cytokines [such as IL-1 $\beta$ , MCP-1 and platelet-derived growth factor (PDGF)] is, in part, responsible for restenosis *via* pro-inflammatory reactions and smooth muscle activation and proliferation (36-38). As reported recently a stent-based release of a selective PDGF-receptor blocker significantly diminished restenosis in a rabbit model (39). Konishi *et al.* showed that activated platelets play an important role in neointimal hyperplasia in mice models (40). Furthermore Rutherford *et al.* reported inhibition of neointimal response to balloon injury in the rat carotid artery using antibodies to PDGF (41). The importance of platelets action in neointimal formation was also proved in humans. Rudez *et al.* showed that common variations in the platelet receptor *P2Y12* gene may serve as a useful marker for restenosis risk stratification (42).

Based on previous observations, we conducted the present study to examine whether low-power laser illumination influences the endothelial inflammatory response (measured by CD54 and MCP-1 expression) and pro-coagulant activity (vWF release) after stimulation with IL-1 $\beta$ .

## Materials and Methods

**Cell culture.** Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical cords by the use of the method described by Jaffe *et al.* (43). Cells were cultured in 25 cm<sup>2</sup> culture flasks (BD Biosciences, Franklin Lakes, NJ, USA) at 37°C under a humid atmosphere with 5% CO<sub>2</sub> (standard conditions). Culture medium-199 (Sigma, Munich, Germany) supplemented with 20% fetal calf serum (FCS) (Cambrex, Verviers, Belgium), 10 UI/ml penicillin (Sigma), 100  $\mu$ g/ml streptomycin (Sigma) and 2 mM L-glutamine (Sigma) was used. When cells reached 80% confluence, endothelial cell monolayer were harvested from the culture flasks and passaged to the 24-well plates (BD Biosciences) using 0.05 % trypsin and EDTA solution (Sigma). Tightly-confluent monolayers were used for the experiments.

To measure the influence of low-power laser influence on inflammatory response, HUVECs were exposed to IL-1 $\beta$  (BioSource, Washington D.C., USA) at 1 ng/ml for 6 and 24 h stimulation followed by laser light illumination.

**Laser source.** For the experiment, a 808-nm wavelength laser diode (LT 808/2000; Laser Secura, Wroclaw, Poland) with a maximal output power of 2 W was used. The laser diode was optically connected to a silica fiber which allowed for homogenous cell culture illumination. Measurements of laser power output before every culture illumination were performed by integrating sphere (detector model 13 PDH 003 and controller model 13 PDC 001, Melles-Griot, Albuquerque, USA). The homogeneity of the laser irradiation was measured with CCD camera (Monacor, Bremen, Germany) (Figure 1). Laser light energy doses of 1.5 and 4.5 J/cm<sup>2</sup> applied at 50mW for 90 and 270 sec were used during the study.

**Cytofluorometry for CD54 and CD62E measurements.** Cell preparation and cytometry measurements were performed according to methods described elsewhere (44). In short, HUVEC monolayers were washed with phosphate-buffered saline (PBS) (pH 7.4) and fixed with 0.5 % formaldehyde for 5 min. Cells were then harvested with the use of a trypsin/EDTA solution and washed with PBS at the end of the process. Immunofluorescence labelling was performed with humanized mouse monoclonal antibodies against CD54 and CD62E conjugated with phycoerythrin (PE) (Becton Dickinson, Franklin Lakes, New Jersey, USA) in separate experiments. HUVECs were incubated for 30 min with the antibodies and washed with PBS at the end of the procedure. Mouse IgG1 PE-conjugated was used as an isotypic control (Becton Dickinson). In our experiment we used a Galaxy cytofluorimeter (Partec Dako, Munster, Germany).

**Enzyme-linked immunosorbent assay (ELISA) for MCP-1.** For the measurement of MCP-1 concentration in the supernatants a commercial ELISA kit (Quantikine-Human MCP-1 Immunoassay; R&D Systems, Minneapolis, Minnesota, USA) was used. Briefly, the HUVECs cell culture supernatant samples were added to a 96-well plate coated with monoclonal antibody specific for MCP-1. After incubation an enzyme-linked polyclonal antibody specific for MCP-1 was added and was followed by a substrate solution. The sensitivity of the test is 5 pg/ml. MCP-1 concentration walls measured in cell culture media in 6 and 24 h after IL-1 $\beta$  (1 ng/ml) stimulation and IL-1 $\beta$  (1 ng/ml) plus laser irradiation (1.5 or 4.5 J/cm<sup>2</sup>).

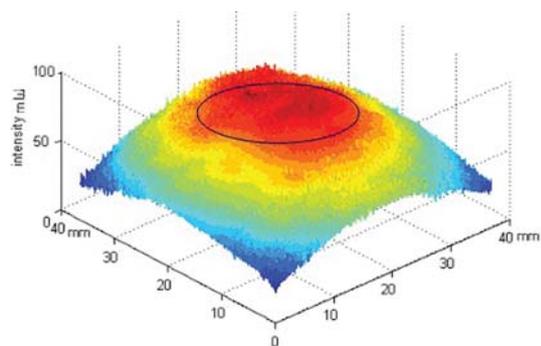


Figure 1. Homogenous distribution of the laser light when illuminating the surface of cultured cells (blue circle).

**Expression of vWF.** vWF activity was determined in supernatants of endothelial cells using a commercial ELISA test (Asserachrom vWF, Diagnostica Stago, Asnières sur Seine, France). vWF was measured as the percentage activity of vWF in cell culture media at 6 and 24 h after IL-1 $\beta$  (1 ng/ml) stimulation, IL-1 $\beta$  (1 ng/ml) plus laser irradiation (1.5 or 4.5 J/cm<sup>2</sup>) or laser illumination alone (1.5 or 4.5 J/cm<sup>2</sup>).

**Cell viability.** In order to estimate HUVEC viability we placed 500  $\mu$ l of each recovered cell suspension tested (controls, laser and/or IL-1 $\beta$  treated groups) in a test tube, added 500  $\mu$ l 0.4% Trypan blue and mixed gently, then we left it stand for 5 min at room temperature. We then placed 10  $\mu$ l of stained cells on the hemocytometer and counted the number of viable (unstained) to dead (stained) cells.

**Statistical analysis.** Results are presented as means $\pm$ SD. The normal distribution of the data was tested with the Kolmogorov-Smirnov test and parametric (Student *t*-test, ANOVA) or non-parametric (Mann-Whitney *U*-test, Friedman ANOVA) tests were used for further analysis. A *p*-value of less than 0.05 was considered significant. All tests were performed with the use of Statistica 7.1 (Statsoft, Cracow, Poland).

## Results

**CD54 and CD62E expression.** CD54 expression on the HUVECs surface (Figure 2a) was significantly enhanced by IL-1 $\beta$  stimulation after 6 and especially after 24 h compared to the untreated controls (*p*<0.05 and *p*<0.05 respectively). Laser light illumination post-IL-1 $\beta$  stimulation with both energies inhibited this response and percentage of cells expressing CD54 on HUVECs were comparable to these on unstimulated cell.

CD62E expression on IL-1 $\beta$  significantly stimulated HUVEC (Figure 2b) compared to control, after 6 and 24 h (*p*<0.01 and *p*<0.001, respectively). Laser light illumination with 1.5 j/cm<sup>2</sup> and 4.5 J/cm<sup>2</sup> significantly diminished this response with respect to IL-1 $\beta$  stimulated HUVECs at 6-hour (*p*<0.01 for both energies) and 24-hour

observations (*p*<0.001 for both energies), although CD62E expression remained higher compared to controls (*p*<0.05 for both energies).

**Expression of MCP-1.** MCP-1 protein concentration in cell culture medium (Figure 3) was enhanced by cell stimulation with 1 ng/ml IL-1 $\beta$  both at 6 and 24 h after stimulation versus control (*p*<0.01 and *p*<0.001 respectively). Laser illumination of 4.5 j/cm<sup>2</sup> significantly inhibited the increase of MCP-1 concentration induced after 6 h of IL-1 $\beta$  stimulation (*p*<0.05) compared to IL-1 $\beta$ -treated cells, although the MCP-1 protein level was still higher compared to that of the control group (*p*<0.05). After 24-h stimulation, the inhibitory effect of laser irradiation disappeared and MCP-1 concentrations were significantly higher with respect to control values for all treatments (*p*<0.001). There were no differences in MCP-1 levels after IL-1 $\beta$  stimulation with 1.5 J/cm<sup>2</sup> illumination compared to a IL-1 $\beta$  stimulation-alone.

**Expression of vWF.** vWF concentration in the supernatant from endothelial cell cultures (Figure 4) was elevated in HUVECs stimulated with IL-1 $\beta$  (1 ng/ml) and reached the highest level after stimulation of 6 h (*p*<0.01 compared to control). Laser illumination with 1.5 J/cm<sup>2</sup> and 4.5 J/cm<sup>2</sup> inhibited the increase of vWF expression after IL-1 $\beta$  stimulation (*p*<0.01 for both energies in respect to stimulated cells). With 24-h IL-1 $\beta$  stimulation, levels of vWF were comparable in all groups.

**Cell viability.** There were from 97% to 99% viable cells in control cultures, and in and laser-and/or IL-1 $\beta$ -treated ones. There were no significant differences in viable cells count between any of the groups tested.

## Discussion

The application of low-power laser light has been recently used for many conditions requiring inflammatory process modulation *e.g.* bone healing, osteoarticular diseases, traumatology, pain control (1-3, 45-47). There were some observations reporting that 632 nm, 650 nm and 808 nm laser light reduces neointima proliferation and restenosis rate after coronary angioplasty procedures (10-12). Little is known about the mode of action of light way in all these processes, but it was postulated that laser irradiation may diminish the inflammatory reaction. In this study, we examined whether laser light modulates the influence of IL-1 $\beta$ , one of the most important factors in the inflammatory cascade, on HUVEC cells. To our knowledge, this is the first study to assess CD54, MCP-1 and vWF expression in HUVECs illuminated with 808 nm laser light.

Our results demonstrate that low-power 808 nm laser irradiation may influence the pro-inflammatory response in

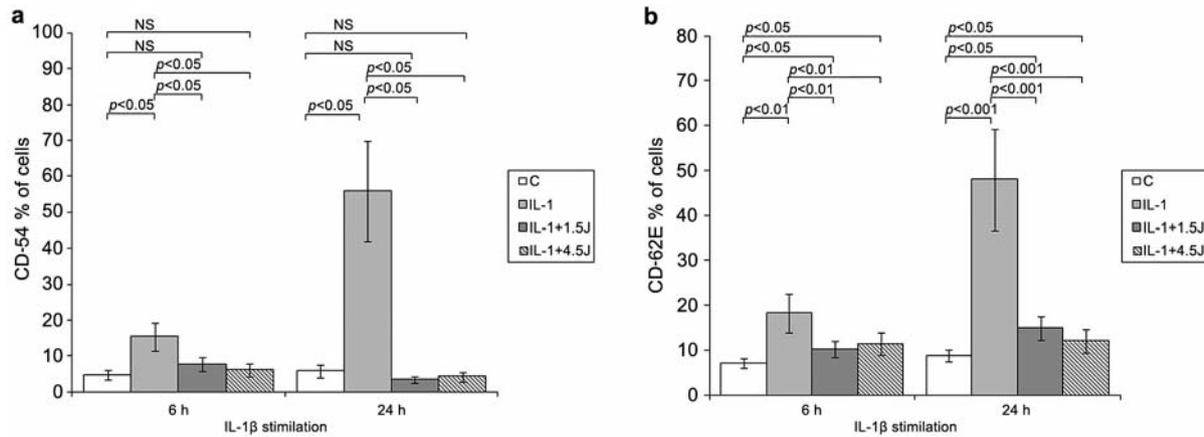


Figure 2. Effect of the laser illumination on CD54 (a) and CD62E (b) concentration. Results are shown for endothelial cell controls (C), cells stimulated with Interleukin-1 $\beta$  (1 ng/ml, IL-1 $\beta$ ), cells stimulated with IL-1 $\beta$  and illuminated with laser light of 1.5 J (IL-1+1.5 J) and 4.5 J (IL-1+4.5 J). Each bar represents the mean and standard deviation of four measurements in two experiments;  $p < 0.05$  was deemed significant.

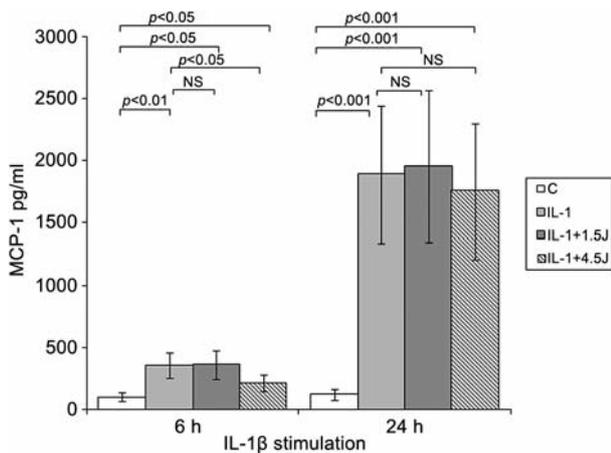


Figure 3. Effect of the laser illumination on MCP-1 concentration. Results are shown for endothelial cell controls (C), cells stimulated with Interleukin-1 $\beta$  (1 ng/ml, IL-1 $\beta$ ), cells stimulated with IL-1 $\beta$  and illuminated with laser light of 1.5 J (IL-1+1.5 J) and 4.5 J (IL-1+4.5 J). Each bar represents the mean and standard deviation of four measurements in two experiments;  $p < 0.05$  was deemed significant.

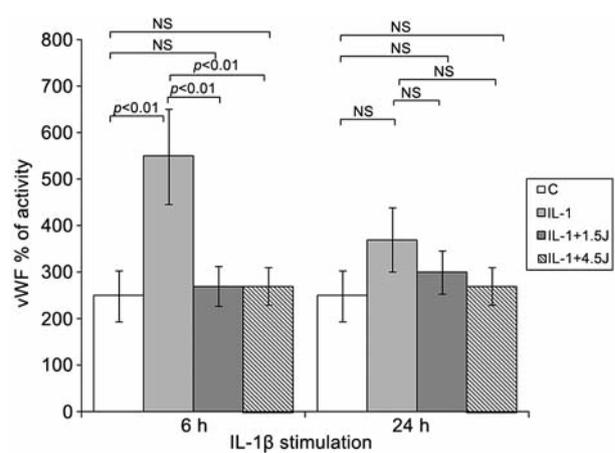


Figure 4. Effect of the laser illumination on von Willebrand Factor (vWF) concentration. Results are shown for endothelial controls (C), cells stimulated with Interleukin-1 $\beta$  (1 ng/ml, IL-1 $\beta$ ), cells stimulated with IL-1 $\beta$  and illuminated with laser light of 1.5 J (IL-1+1.5 J) and 4.5 J (IL-1+4.5 J). Each bar represents the mean and standard deviation of four measurements in two experiments;  $p < 0.05$  was deemed significant.

HUVECs stimulated with IL-1 $\beta$ . We observed that laser irradiation diminishes CD54 expression on the cell membrane and MCP-1 concentration in cell supernatants after IL-1 $\beta$  stimulation. It was previously shown that low-power laser light reverses histamine-induced arterial spasm (48), inhibits smooth muscle cells (SMC) migration (49), stimulates inducible nitric oxide synthase (iNOS) activation (5) and enhances endothelial repair after balloon denudation (50), which finally leads to reduction of neointima formation and restenosis rate both in animal models and humans (10-12).

The diminution of CD54 presence on IL-1 $\beta$ -stimulated cells after low-power 808 nm laser irradiation may indicate that the inhibition of the inflammatory response is another way by which laser illumination prevents restenosis *in vivo*. An anti-inflammatory mechanism of restenosis prevention with laser light is even more probable as we also observed a markedly diminished MCP-1 concentration in HUVEC supernatant from HUVEC cultures after laser irradiation.

The present study also shows that laser illumination may diminish pro-coagulant activity in endothelial cells

stimulated with IL-1 $\beta$ . Both doses of laser irradiation reduced vWF release from HUVECs. Our data show that laser light may be an important factor for the control of the platelet activation cascade, as a lower level of vWF in plasma may lead to inhibition of platelet adhesion and to lower factor Xa generation *via* diminution of factor VIIIa availability.

The observation of the modulation of IL-1 $\beta$  action with low-power 808 nm laser illumination may be crucial and may justify the application of laser light in conditions of inflammatory response and repair processes. The mode of action of low-power laser light and its influence on intracellular interactions has not yet been well-explained. In previous studies, it was postulated that laser illumination causes changes in mitochondrial respiration and ATP synthesis, reduction of oxidative stress or modulation of membrane component activity (51-54). If laser irradiation does influence inflammatory processes, its usage in the acceleration of wound healing or modulation of osteoarticular diseases may be of considerable interest. Our observation may be also important in restenosis prevention since inflammatory reactions, involving migration of leucocytes and cytokine release, play a crucial role in early stages of the restenosis cascade. The latter leads to formation of neointima composed of smooth muscle cells and extracellular matrix, a process similar to wound healing in other tissues.

In summary low-power 808 nm laser irradiation influences pro-inflammatory activity in cultured endothelial cells. Taking into account that inflammation plays a pivotal role in neointimal hyperplasia, this effect may, at least in part, explain the previously observed reduction in restenosis rate after laser light illumination in humans and in animal models.

## Conclusion

Low-power laser illumination diminishes pro-inflammatory and pro-coagulant activity of IL-1 $\beta$ -stimulated HUVECs. Given the findings of this study, we conclude, that one of the possible mechanisms underlying the positive influence of laser light on healing, regeneration and reduction of restenosis rate observed after coronary angioplasty, may be the inhibition of inflammatory processes.

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