INTRODUCTION

Percutaneous coronary intervention (PCI) is now routinely used for revascularization of occluded coronary arteries. Recent meta-analyses indicate that intensive short-term pre-treatment with statins may significantly reduce peri-procedural complications and adverse events in patients undergoing PCI (1-4). The mechanism whereby statins exert this effect is not clear, but appears to be independent of cholesterol lowering. More likely it is related to anti-inflammatory properties of statins and their ability to alleviate PCI-associated endothelial cell activation (5). It has been demonstrated that statins improve endothelial cell-dependent coronary blood flow (6, 7) and reduce endothelial cell expression of adhesion molecules (8) and pro-inflammatory cytokines (9). In vitro and animal studies indicate that the beneficial effect of statins may also be related to increased bioavailability of nitric oxide (10, 11).

In addition, statins are known to recruit bone marrow-derived endothelial progenitor cells (EPC) (12). In this respect, recent study has demonstrated that intensive therapy with atorvastatin initiated before PCI effectively mobilizes EPC and increases their attachment to stent struts (13). This effect is of importance as PCI is associated with endothelial cell injury and prompt re-endothelialization reduces neointimal hyperplasia and subsequent restenosis (14). However, the effect of statins on endothelial cell regeneration may be more complex. This is because healing of endothelial cell wounds occurs not only through EPC incorporation, but also through proliferation and migration of neighboring cells (15). The impact of statins on these processes is less clear and appears to depend critically on the dose applied (16). At low concentrations statins stimulate endothelial cell proliferation and migration, while high concentrations act to the contrary (17). In the clinical setting both effects could be viewed as beneficial, since stimulation of cell proliferation would accelerate endothelial cell recovery from PCI-induced injuries, but on the other hand, the inhibition of endothelial cell growth within atherosclerotic lesions would decrease the risk of plaque rupture. To strike the right balance between these effects, both timing and dosage of therapy need to be carefully optimized so that the plaque stability is improved without compromising post-PCI re-endothelialization.

Most clinical experience on peri-procedural statin administration was gained from studies with atorvastatin (4). Pre-treatment with atorvastatin showed benefits both in patients with stable angina undergoing elective PCI (18) and in patients with acute coronary syndromes undergoing early PCI (19). We have therefore focused on atorvastatin and set out to examine how acute exposure to atorvastatin at clinically relevant doses affects vascular endothelial cell wound healing independent of EPC. To this end we analyzed recovery of
cultured endothelial cells from scratch injuries mimicking those occurring during PCI.

MATERIAL AND METHODS

Unless indicated otherwise, all reagents were from Sigma-Aldrich. Cell culture plastics were from Nunc and Costar. The study protocol was accepted by local Ethical Committee at Poznan Medical University.

Cell culture

The experiments were performed using human umbilical vein endothelial cells (HUVEC) of the EA.hy926 line (kindly provided by Dr. CJ Edgell, University of North Carolina, Chapel Hill, USA) (20). Cells were routinely maintained in Earl’s-buffered M199 culture medium, supplemented with amphotericin (2.5 µg/mL), gentamycin (50 µg/mL), L-glutamine (2 mM), hydrocortisone (0.4 µg/mL), and 10% v/v fetal calf serum (Invitrogen).

Atorvastatin exposure

Atorvastatin was kindly donated by Pfizer and dissolved in dimethyl sulfoxide (DMSO) (21, 22). Atorvastatin concentrations of 0.01–0.1 µM that were used throughout the study corresponded to the levels found in serum after oral administration of 40 mg atorvastatin (23). Final DMSO concentration in test media was 0.2% (v/v) and the same concentration was applied to the controls. Preliminary experiments have determined that this DMSO concentration did not impair HUVEC viability, as assessed by the MTT test (data not shown).

Cell proliferation

Cell proliferation was assessed by [3H]-thymidine incorporation. Briefly, cells were plated at a density of 2×10^4 cells/cm², allowed to attach for 4 hours and then treated for 24 hours with either atorvastatin or vehicle control in the presence of [3H]-thymidine (1 µCi/ml; Institute of Radioisotopes, Prague, Czech Republic). After the incubation the cells were harvested, precipitated with 10% (w/v) trichloroacetic acid, and dissolved in 0.1 M NaOH. The radioactivity released was measured in a beta liquid scintillation counter (Wallac Perkin Elmer).

Cell migration

Cell migration was assessed with the use of QCM™ Chemotaxis 96-well Cell Migration Assay with 8 µm pore size membranes (Chemicon/Milipore). Cells at <80% confluence were incubated in the presence of either atorvastatin or vehicle control for 24 hours and then rendered quiescent by serum reduction (to 0.1%) for the next 24 hours. After that the cells were harvested, washed, re-suspended in serum-free medium and placed in a migration chamber (5×10^4 cells/100 µl). Cells were then stimulated for 24 hours with standard 10% serum-containing medium with or without atorvastatin. Migrated cells were detached and treated for 15 minutes with the CyQuant GR dye in the lysis buffer, as per manufacturer’s instructions. Fluorescence of cell lysates was measured with a fluorescence microplate reader (Perkin Elmer) using 480 nm and 520 nm wavelengths for excitation and emission, respectively.

Cell viability

Viability of cells following a 24-hour exposure to atorvastatin was assessed with the MTT assay. It measures the metabolic conversion of the MTT salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolinum bromide) by active mitochondrial dehydrogenases (24). The assay was performed as described previously (25). Briefly, after exposure to atorvastatin, the cells were treated with MTT (1.25 mg/ml) for 4 hours at 37°C. The formazan product generated was solubilized by the addition of 20% sodium dodecyl sulfate and 50% N,N-dimethylformamide, and quantified by measuring its absorbance at 595 nm.

Wounding healing

Cells were grown to confluence, pre-treated with or without atorvastatin for 24 hours and then scratched with a cell scraper (Nunc). The resulting debris was removed by gentle washing with medium. After that the cells were placed in an incubator coupled to an Axio Observer D1 inverted microscope (Zeiss). Cells were maintained for up to 12 hours in standard culture conditions.

![Fig. 1. Effect of atorvastatin on endothelial cell viability. HUVEC were treated with increasing doses of atorvastatin or vehicle control for 1, 2, and 6 days. After the exposure cell viability was measured with the MTT test (n=3). Data were expressed as a percentage of control at the same time point. Asterisks represent a significant difference compared to a respective control.](image-url)
medium with either atorvastatin or vehicle control. The images of the closing wound were acquired by time-lapse microscopy at 30-minute intervals and analyzed using the AxioVision Rel. 4.6.3 image analysis software (Zeiss).

**Cytochemistry**

All cytochemical procedures were performed on cells cultured in Lab-Tek Chamber Slides (Nunc). Incorporation of bromodeoxyuridine (BrdU) was visualized using a Zymed® BrdU Staining kit (Invitrogen), as per manufacturer’s instructions.

**Cytokine measurements**

Release of selected cytokines (IL-6, IL-8, MCP-1), adhesion molecules (sICAM-1), and extracellular matrix proteins (fibronectin) by HUVEC was assessed under basal conditions (constitutive release) and following the stimulation with IL-1β (1 ng/ml) and TNF-α (10 ng/ml). Target molecules were measured with appropriate DuoSet Immunoassay Kits (R&D Systems). The assays were designed and performed according to the manufacturer’s instructions. Fibronectin was measured as previously described (26). The results were normalized per µg of cell protein. Protein concentration in cell lysates was determined with the Bradford method (27) using the Protein Assay Dye Reagent (Bio-Rad).

**Fig. 2.** Effect of atorvastatin on endothelial cell proliferation. Proliferation of HUVEC treated with increasing doses of atorvastatin was assessed over a 24-hour period by "H-thymidine incorporation (n=8). Asterisks represent a significant difference compared to control cells.

**Fig. 3.** Effect of atorvastatin on endothelial cell wound healing. (A): Exemplary microphotographs of wound closure in control HUVEC; magnification 100×; (B): Kinetics of wound healing in cells pre-treated for 24 hour and then incubated in the presence or absence of atorvastatin (n=13).
Fig. 4. Effect of atorvastatin on bromodeoxyuridine incorporation by endothelial cells before and after scratch injury. Exemplary microphotographs of BrdU labeling in control cells before (A) and after injury (B); magnification 100×; the arrows indicate typical cells staining positively for BrdU. (C): Cells were pre-treated with atorvastatin for 24 hours and then wounded. Percentage of cells incorporating BrdU was assessed in each group immediately before and 4 hours after injury (n=7).

Fig. 5. Effect of atorvastatin on endothelial cell migration. HUVEC were treated with atorvastatin as described in the Methods and assessed for the capacity to migrate in the presence or absence of stimulation (n=10).
Table 1. Effect of atorvastatin on the release of mediators by endothelial cells. Mediators secreted were measured in post-culture supernatants following a 24-hour exposure to atorvastatin (n=7) in the presence or absence of stimulation with IL-1β (1 ng/ml) and TNF-α (10 ng/ml). Asterisks represent a significant difference compared to control cells treated with the vehicle only.

<table>
<thead>
<tr>
<th>Mediator release (pg/µg cell protein)</th>
<th>Constitutive release</th>
<th>IL-1β+TNF-α-stimulated release</th>
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<tr>
<td></td>
<td>Atorvastatin (µM)</td>
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<td>0</td>
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<td>IL-6</td>
<td>0.5 ± 0.2</td>
<td>0.3 ± 0.1</td>
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<td>IL-8</td>
<td>1.2 ± 0.3</td>
<td>0.7 ± 0.3</td>
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<td>MCP-1</td>
<td>2.2 ± 1.0</td>
<td>1.7 ± 0.7*</td>
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<tr>
<td>sICAM-1</td>
<td>1.4 ± 0.5</td>
<td>1.0 ± 0.4*</td>
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<tr>
<td>Fibronectin</td>
<td>5.5 ± 2.3</td>
<td>3.6 ± 1.9</td>
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Statistical analysis

The data were analyzed with repeated measures analysis of variance using GraphPrism™ 5.00 software (GraphPad Software Inc.). A p value of less than 0.05 was considered significant. Results are presented as means ±S.D.

RESULTS

Effect of atorvastatin on endothelial cell proliferation and viability

Exposure of HUVEC to atorvastatin resulted in a dose-dependent inhibition of cell viability as measured by the MTT assay (Fig. 1). After 24 hours the effect was evident for 10 µM atorvastatin, and after prolonged incubation also for 1 µM. Atorvastatin at doses ≥ 1 µM inhibited also cell proliferation (Fig. 2). At the highest dose of atorvastatin tested (10 µM) HUVEC proliferation was 64 ±3% of the control. In contrast, atorvastatin at clinically relevant doses of 0.01 and 0.1 µM impaired neither HUVEC viability proliferation. These doses were used in further experiments.

Effect of atorvastatin on endothelial cell wound closure

Scratch wounds were inflicted on cells pre-treated with or without atorvastatin for 24 hours. The surface area of the wounds generated did not differ between the groups and was 444 ±50 mm², 402 ±55 mm², and 419 ±42 mm² for cells exposed to vehicle control, 0.01 µM and 0.1 µM of atorvastatin, respectively. Cells from all groups repopulated the denuded areas within 12 hours and there was no difference in the kinetics of the process between cells treated with or without atorvastatin (Fig. 3).

To assess whether atorvastatin changed the contribution of cell proliferation to wound repair, endothelial cell monolayers were tested for incorporation of BrdU immediately before and 4 hours after a scratch injury (Fig. 4). The fraction of control cells that stained for BrdU was 35 ±8% before and 31 ±2% after injury. Pre-treatment of HUVEC with atorvastatin at 0.01 µM and 0.1 µM did not significantly change these percentages.

Effect of atorvastatin on endothelial cell migration

Compared to the control, atorvastatin at doses up to 0.1 µM did not impair HUVEC migration in the absence of stimulation (Fig. 5). Following the stimulation with serum migration of control HUVEC increased to 208±76% of the baseline values. For cells exposed to 0.01 µM and 0.1 µM atorvastatin these values were 210±72% and 181±41%, respectively, and did not differ significantly from the controls.

Effect of atorvastatin on endothelial cell cytokine production

Exposure of HUVEC to atorvastatin resulted in a dose-dependent decrease in both the constitutive and the cytokine-stimulated release of IL-6, IL-8, MCP-1, sICAM-1, and fibronectin (Table 1). For MCP-1 and sICAM-1 the effect became evident with atorvastatin at 0.01 µM, for all other mediators it was significant with the dose of 0.1 µM.

DISSCUSION

Statins display pleiotropic properties and exert their benefits partly through the inhibition of vascular smooth muscle cell (VSMC) proliferation (28). This effect is important for the prevention of post-PCI restenosis. Several studies have demonstrated that statins reduce VSMC proliferation and migration *in vitro* (28-33). The concentrations of atorvastatin that produced this effect approximated those found in serum after oral administration of average therapeutic doses. However, Axel et al. observed that the same doses may inhibit the growth of endothelial cells to a significantly greater extent compared to VSMC (29). In contrast, Giordano et al. have recently demonstrated that statins at doses that effectively inhibited VSMC proliferation and migration did not impair these processes in endothelial cells (34). Jaschke et al. (35) observed a similar effect in cells treated with cerivastatin. They have also demonstrated that the implantation of stents coated with cerivastatin did not impair re-endothelialization but inhibited the neoointima formation (35). Our data seem to be in keeping with these observations and confirm that atorvastatin at doses up to 0.1 µM does not delay endothelial cell wound closure and does not hamper endothelial cell proliferation and migration. These findings are of clinical significance as the integrity of the endothelial barrier protects against excessive VSMC growth (36). Failure of the denuded surfaces to re-endothelialize leads to increased accumulation of VSMC and the neoointima formation (37). Urbich et al. (16) have titrated the effect of atorvastatin to find that it promoted endothelial cell migration at low doses (0.001–0.01 µM), did not affect it at moderate doses (0.1 µM), but inhibited it at high doses (1 µM). The mechanism underlying this biphasic effect of statins on endothelial cell proliferation and migration has been attributed to their impact on protein prenylation. It appears that at low doses statins inhibit cholesterol synthesis, but do not impair the biosynthesis of farnesyl and geranylgeranyl pyrophosphates that are key intermediates in the pathways controlling cell growth. In contrast, high doses of statins inhibit both the cholesterol synthesis and the synthesis of prenyl radicals, which results in the inhibition of cell proliferation and migration (5, 38). Similar mechanisms may underlie anti-inflammatory effects of statins toward endothelial cells (5, 39). Statins (including
atorvastatin at high doses) were found to inhibit production of MCP-1 and IL-8 (40, 41) and these effects were attributed to the inhibition of protein prenylation (42). On the other hand, Dayoub et al. (43) reported that atorvastatin at doses as high as 1 μM failed to inhibit the release of IL-6 from endothelial cells exposed to lipopolysaccharide. Interestingly, we have observed that even at low concentrations atorvastatin reduced the constitutive and stimulated production of inflammatory cytokines, adhesion molecules, and fibronectin. Adding to the complexity of this issue, it appears that the final effect of statins in vivo may depend on the exact clinical setting. For example, it has been demonstrated that simvastatin decreased the release of pro-inflammatory cytokines by monocytes from patients with isolated hypercholesterolemia but not from those with impaired glucose tolerance (44).

Several studies reported also on down-regulation by stations of endothelial cell ICAM-1 (39). This effect was reversed by mevalonate, and linked to the increased NO production (45). Indeed, it has been demonstrated that statins are capable of increasing the expression and activity of endothelial nitric oxide synthase (eNOS) in HUVEC (46). The inhibition by atorvastatin of fibronectin secretion also may bear clinical significance as fibronectin has been implicated in the formation of atherosclerotic plaques and expansion of VSMC (47). An additional benefit of atorvastatin in the context of acute coronary syndromes could be related to its anti-arrhythmic properties. It has been observed in an experimental rat model that atorvastatin decreased the propensity for ventricular arrhythmias, possibly by stabilizing the cardiomyocyte cell-to-cell junction integrity (48).

Given the clinical background of our study, the application of HUVEC rather than coronary artery endothelial cells may be seen as a limitation. However, gene expression profiling revealed that, apart from some genes expressed preferentially in the arteries, cells from these two locations shared a lot of similarities (49). Some reservations may also be raised against the usage of immortalized HUVEC line rather than primary HUVEC. It has been demonstrated that the pattern of gene expression in unstimulated and atorvastatin-treated EA.hy926 cells matched that in primary HUVEC, although some rather expected differences in the expression of genes controlling cell cycle were observed (50). Therefore, additional studies that would validate our observations with primary coronary artery endothelial cells are warranted.

In summary, the present study shows that atorvastatin at clinically relevant concentrations does not impair endothelial cell wound healing but is capable of curtailing the production of pro-inflammatory cytokines. Our data indicate that atorvastatin at these doses is safe to use after PCI as it will not delay endothelial cell recovery from injuries. In addition, it may dampen the inflammatory response associated with the procedure.

Abbreviations: BrdU – bromodeoxyuridine; DMSO – dimethyl sulfoxide; eNOS – endothelial nitric oxide synthase; EPC – endothelial progenitor cells; HUVEC – human umbilical vein endothelial cells, IL – interleukin; MCP-1 – monocyte chemoattractant protein-1; MTT – 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; PCI – percutaneous coronary intervention; RFU – relative fluorescence units; sICAM-1 – soluble intercellular adhesion molecule-1; TNF-α – tumor necrosis factor-α; VSMC – vascular smooth muscle cells

Conflict of interests: None declared.

REFERENCES


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