Endothelial cell-derived microparticles
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Chapter 5

Simvastatin-induced endothelial cell detachment and microparticle release are prenylation dependent

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ABSTRACT

Background: Statins reduce cardiovascular disease risk and affect endothelial function by cholesterol-dependent and independent mechanisms. Recently, circulating (detached) endothelial cells and endothelial microparticles (EMP) have been associated with endothelial functions in vitro and in vivo.

Objective: We investigated whether simvastatin affects endothelial detachment and release of EMP.

Methods: Human umbilical vein endothelial cells (HUVEC) were incubated with clinically relevant concentrations of simvastatin (1.0 and 5.0 μmol/L), with or without mevalonic acid (100 μmol/L) or geranylgeranylpyrophosphate (GGPP; 20 μmol/L) for 24 hours, and analyzed by flow cytometry and Western blot.

Results: Simvastatin increased detachment from 12.5% ± 4.1 to 26.0% ± 7.6 (1.0 μmol/L; P=0.013) and 28.9% ± 2.2 (5.0 μmol/L; P=0.002). Concurrently, EMP release increased 2.5-fold (P=0.098 and P=0.041, respectively). Adherent cells showed no signs of simvastatin-induced apoptosis (caspase 3, annexin V, propidium iodide), suggesting that cell detachment and EMP release are not necessarily due to apoptosis. In contrast, the majority of detached cells was apoptotic, although the fraction of detached cells that showed signs of apoptosis (>70%) was unaffected by simvastatin. Similar to these detached cells, EMP contained caspase 3. Furthermore, detached cells and EMP contained caspase 8 but not caspase 9. By restoring either cholesterol biosynthesis and prenylation (mevalonate) or prenylation alone (GGPP), all simvastatin-induced effects on detachment and EMP release could be reversed.

Conclusions: Simvastatin promotes detachment and EMP release by inhibiting prenylation, presumably via a caspase 8-dependent mechanism. We hypothesize that by facilitating detachment and EMP release, statins may improve the overall condition of the vascular endothelium.
INTRODUCTION

Statins are widely prescribed lipid-lowering drugs that significantly reduce cardiovascular morbidity and mortality in many different patient populations, as demonstrated in multiple large primary and secondary prevention trials [1-9]. By inhibiting 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of cholesterol biosynthesis, statins reduce both total and low-density lipoprotein-associated cholesterol (LDL-associated cholesterol). Their beneficial effects in cardiovascular disease (CVD) patients have been largely attributed to their efficacy to lower LDL-associated cholesterol [4]. Statins, also have additional pleiotropic (cholesterol-independent) effects, many of which are mediated by the vascular endothelium [10-17]. However, data from several, mainly in vitro studies, may be difficult to interpret since statins were used at pharmacological and possibly cytotoxic concentrations or in combination with a variety of agonists like TNF-α, endotoxin or thrombin [13,16]. The existence of pleiotropic effects of statins in vivo, separate from their cholesterol-lowering potential, was recently substantiated by Landmesser et al, who showed improvement of endothelial dysfunction in patients with chronic heart failure after simvastatin therapy but not after treatment with the cholesterol absorption inhibitor ezetimibe, given at a dose that lowered LDL-associated cholesterol to a similar extent [18].

Pleiotropic effects of statins seem to be mainly caused by inhibition of protein prenylation. Prenylation is a post-translational mechanism of protein modification, in which intermediates of the mevalonate pathway, like geranylgeranylpyrophosphate (GGPP), are attached to proteins. Geranylgeranylated proteins, including the small G proteins Rho, Rac and Rab, bind to cell membranes and are required for transmembrane signaling [19]. As a consequence, G proteins are involved in the regulation of cell growth, differentiation, gene expression, cytoskeletal assembly and cell motility, formation of microparticles (MP) or “apoptotic bodies”, protein and lipid trafficking, nuclear transport and host defense [19]. Thus, by preventing formation of mevalonate, statins block cholesterol biosynthesis and transmembrane signaling.

Previously, we showed that cultures of viable and unstimulated human umbilical vein endothelial cells (HUVEC) contain small numbers of detached cells (‘floaters’) undergoing
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apoptosis as well as endothelial cell-derived MP (EMP) [20]. Since in the afore-
mentioned in vitro studies no or hardly any attention was paid to detachment and/or
release of EMP, we hypothesized that to gain insight into the full response of endothelial
cells to statins, not only the adherent cell fraction but also the corresponding fraction of
detached cells and EMP have to be analyzed. Therefore, in order to study the true impact
of statins on the endothelium, we determined the effect of simvastatin on human
endothelial cells by concurrently analyzing adherent cells, detached cells and EMP.

MATERIALS AND METHODS

Reagents and assays

Medium M199, penicillin, streptomycin, Isocove’s modified dulbecco’s medium and
L-glutamine were obtained from GibcoBRL, Life Technologies (Paisley, Scotland).
Human serum and fetal calf serum (both heat inactivated during 30 minutes at 56 ºC; HuSi
and FCSi, respectively) were from BioWhittaker (Walkersville, MD). Human serum
albumin (HSA) was obtained from Sanquin (Amsterdam, The Netherlands). Human
recombinant basic fibroblast growth factor (bFGF), and epidermal growth factor (EGF)
were obtained from Invitrogen life technologies (Carlsbad, CA). Collagenase (type 1A),
geranylgeranylpynrophosphate (GGPP), mevalonolactone (mevalonate) and propidium
iodide (PI) were from Sigma (St. Louis, MO). APC-labeled annexin V was from Caltag
Laboratories (Burlingame, CA). Heparin (400 U/mL) was from Leo Pharma BV (Breda,
The Netherlands), trypsin from Difco Laboratories (Detroit, MI), and simvastatin from
Calbiochem (Darmstadt, Germany). Tissue culture flasks were from Greiner Labortechnik
(Frickenhausen, Germany) and gelatin from Difco Laboratories (Sparks, MD). Stock
solutions of simvastatin, mevalonate and GGPP were prepared in ethanol, ethanol and
methanol, respectively. Antibodies against (pro-)caspase 9, (pro-)caspase 8 and caspase 3
for Western blotting were obtained from Cell Signaling (Beverly, MA). Anti-procaspase 3
was from Transduction Laboratories (San Diego, CA). Secondary antibodies used for
Western blot, i.e. goat-anti-mouse HRP conjugate and anti-rabbit HRP conjugate, were
from Biorad (Hercules, CA) and Promega (Madison, USA), respectively. FITC-labeled
annexin V was from Immuno Quality Products (Groningen, The Netherlands).
Isolation, culture and treatment of HUVEC

HUVEC were collected from human umbilical cord veins as described previously [20]. Briefly, umbilical cords were digested with collagenase for 20 minutes at 37 °C. Detached cells were obtained by perfusion of the umbilical cord with medium M199 supplemented with 10% HuSi. The cell suspension was centrifuged for 10 minutes at 180 g and 20 °C, and cells were resuspended in culture medium. HUVEC were cultured in tissue culture flasks coated with 0.75% gelatin (passage 0). Upon confluency at passage 3, HUVEC were kept for 3-4 days in a resting state. Then, the culture supernatant was refreshed and (where indicated) cultures were treated for 24 hours without any addition (control), ethanol (0.2% v/v), methanol (0.2% v/v), ethanol and methanol (both 0.2% v/v), simvastatin (1.0 μM and 5.0 μM final concentration (fc)), mevalonate (100 μM fc), GGPP (20 μM fc), and combinations of simvastatin plus mevalonate or GGPP. Administration of 10-40 mg simvastatin results in (peak) plasma concentrations of 1-6 ng/mL, which is in line with the 0.4-2.1 ng/mL used in our present study [21,22].

Flow cytometric analysis of endothelial cells

Conditioned media (10 mL per 75 cm² flasks) were harvested after 24 hours. First, media were centrifuged for 10 minutes at 180 g and 20 °C to isolate detached endothelial cells and to obtain the cell-free conditioned medium for EMP isolation. The detached cell pellets were carefully resuspended in 1% FCSi in PBS (pH 7.4). In parallel, the adherent endothelial cells were harvested by trypsinization. After 4 minutes, trypsin was neutralized by PBS/FCSi. Both cell suspensions were separately centrifuged for 10 minutes at 180 g and 4 °C, resuspended in PBS/FCSi, kept on melting ice for 15 minutes, and then again centrifuged for 10 minutes at 180 g and 4 °C. The detached cells were resuspended in 0.5 mL PBS/FCSi and the adherent cells in 1 mL PBS/FCSi. For intracellular staining for caspase 3, the active caspase 3-FITC MoAb apoptosis kit I was used (BD Pharmingen; San Diego, CA). From the before mentioned suspension of detached and adherent cells, 100 μL were diluted with 1 mL of ice-cold PBS (pH 7.4). This suspension was centrifuged for 10 minutes at 180 g. After removal of the supernatant, the cells were again diluted with 1 mL of ice-cold PBS and pelleted (10 minutes at 180 g). After removal of the supernatant, cells were resuspended in 500 μL
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cytofix/cytoperm and incubated for 20 minutes on melting ice. To remove the
cytofix/cytoperm, cells were pelleted (10 minutes at 180 g) and supernatant was removed.
Then the cells were washed twice with 10-fold diluted perm/wash, and finally
resuspended in 100 μL 10-fold diluted perm/wash. From this suspension, two aliquots of
50 μL each were incubated for 30 minutes at room temperature with either anti-caspase 3-
FITC (5 μL) or Ig-FITC (5 μL). After incubation, 1 mL of 10-fold diluted perm/wash was
added to each aliquot, and the suspension was centrifuged for 10 minutes at 180 g. The
supernatant was removed and the pellets were resuspended in 300 μL 10-fold diluted
perm/wash. All samples were analyzed in a FACSCalibur flow cytometer (BD; San Jose,
CA). Percentages of adherent and detached cells were compared to the total cell count
(i.e. adherent plus detached cells)/culture flask (100%). Labeling with annexin V and PI to
determine the apoptosis status of the endothelial cells was performed as described
previously [20].

Isolation of EMP

Aliquots (1 mL) of the cell-free conditioned media were frozen in liquid nitrogen and
then stored at – 80 °C. Before use, samples were thawed on melting ice for 1 hour, and
then centrifuged for 1 hour at 17,570 g and 20 °C. Then, 950 μL of (MP-free) supernatant
was removed. The remaining 50 μL of EMP suspension was divided into two aliquots of
25 μL each, of which one aliquot was used for regular flow cytometry and the other
aliquot for intravesicular caspase 3 staining.

For regular flow cytometry, 25 μL EMP suspension was diluted with 225 μL PBS
(154 mmol/L NaCl, 1.4 mmol/L phosphate) containing 10.9 mmol/L trisodium citrate.
EMP were resuspended and again centrifuged for 30 minutes at 17,570 g and 20 °C.
Again, 225 μL of supernatant was removed and EMP (25 μL) were finally diluted with 25
μL PBS/citrate buffer. For intravesicular staining of caspase 3, the 25 μL EMP suspension
was diluted with 225 μL 100-fold diluted perm/wash. EMP were resuspended and
centrifuged for 30 minutes at 17,570 g and 20 °C. Again, 225 μL of supernatant was
removed and EMP (25 μL) were diluted with 25 μL 100-fold diluted perm/wash.
Flow cytometric analysis of EMP

EMP samples were analyzed in a FACSCalibur flow cytometer (BD; San Jose, CA). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain and EMP were characterized as described previously by binding of annexin V. EMP (5 μL aliquots) were diluted with 45 μL PBS containing 2.5 mmol/L CaCl₂ (pH 7.4). Annexin V-APC (5 μL of 20-fold diluted) was added. In the control samples of the MP, annexin V-positive events were identified by placing a threshold in a MP sample (5 μL) diluted with PBS containing 10.9 mmol/L trisodium citrate (45 μL; pH 7.4) and 5 μL of annexin V, i.e. without Ca²⁺. The mixture of MP and annexin V was then incubated for 15 minutes in the dark at room temperature, and finally diluted with 900 μL PBS containing either calcium or citrate. For intravesicular staining of caspase 3, EMP were incubated for 30 minutes with the indicated antibodies and APC-labeled annexin V in the dark at room temperature. The labeling was stopped by addition of 900 μL of 100-fold diluted perm/wash before flow cytometric analysis.

Western blotting

For Western blotting experiments, 400 μL of the detached cell suspensions and 450 μL of the adherent cell suspension were diluted with 5-fold concentrated sample buffer containing β-mercaptoethanol. EMP were harvested by centrifugation from 5 mL of cell-free conditioned medium, and finally resuspended in a mixture of 24 μL PBS and 6 μL 5-fold concentrated sample buffer. Before electrophoresis, all samples were heated for 5 minutes at 100 °C. Electrophoresis was carried out on 8-16% gradient polyacrylamide gel (Biorad; Hercules, CA). The proteins were transferred to PVDF membrane (Biorad). Blots were incubated for 1 hour at room temperature with blocking buffer (Tris-buffered saline-Tween (TBST); 10 mmol/L Tris-HCl, 150 mmol/L NaCl, 0.05% (v/v) Tween-20; pH 7.4), containing 5% (w/v) dry milk powder (Protifar; Nutricia, Vienna, Austria). The blots were subsequently incubated with anti-caspase 3 (1:1,000 v/v), anti-(pro)-caspase 8 (1:1,000 v/v) or anti-(pro)caspase 9 (1:1,000 v/v) for 24 hours at 4 °C, followed by incubation with either anti-rabbit IgG-HRP conjugate (1:7,500 v/v; used in combination with the anti-caspase 3 antibody) or goat-anti-mouse HRP conjugate (1:3,000 v/v; used in combination with the other mentioned antibodies) for 1 hour at room temperature. After each
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incubation step, PVDF membranes were washed three times with TBST for 5-10 minutes. All antibodies were diluted with blocking buffer. The bands were detected using Lum-Light Plus Western Blotting Substrate (Roche; Mannheim, Germany) and exposed to Fuji Medical X-ray film.

Statistical analysis

Data were analyzed with GraphPad Prism for Windows (release 3.02; San Diego, CA). Differences were analyzed by t-test for independent samples and were considered to be significant at P<0.05. All data are presented as mean ± SD. Data were obtained from at least 3 independent experiments, i.e. using endothelial cell cultures from 3 or more different umbilical veins. Data were compared to ethanol- (EtOH) and methanol (MeOH)-treated endothelial cell cultures.

RESULTS

Simvastatin induces endothelial cell detachment

Upon incubation with simvastatin, the detached cell fraction (Figure 1A) increased from 12.5% ± 4.1 (ethanol plus methanol control) to 26.0% ± 7.6 (1.0 μmol/L simvastatin; P=0.013) and 28.9% ± 2.2 (5.0 μmol/L simvastatin; P=0.002). Cell detachment was not affected by incubation with mevalonate (100 μmol/L; P=0.207) or GGPP (20 μmol/L; P=0.205) alone, but both compounds completely prevented simvastatin-induced detachment. Thus, simvastatin-induced endothelial cell detachment can be reversed not only by restoring cholesterol biosynthesis (mevalonate) but also by restoring prenylation (GGPP).

Caspase 3 in adherent and detached endothelial cell fractions

Figure 1B shows flow cytometry dot plots of caspase 3 in adherent (Figure 1B; A-D) and detached (Figure 1B; E-H) endothelial cells. Compared to staining with control antibody (IgG-FITC), a negligible number of adherent cells stained for caspase 3 (Figure 1B; B versus A), and this was similar in the presence of 1.0 or 5.0 μmol/L simvastatin (Figure 1B; C and D, respectively). In contrast, a substantial number of detached cells
stained for caspase 3 (Figure 1B; F versus E), and these numbers increased 2.5 to 3-fold in the presence of either 1.0 or 5.0 μmol/L (Figure 1B; G and H, respectively) simvastatin.

Figure 1. A. Percentage of detached cell after HUVEC were incubated (24 hours) without any additions (control), ethanol (0.2% v/v) plus methanol (0.2% v/v), simvastatin (1.0 μmol/L and 5.0 μmol/L), mevalonate (100 μmol/L) without or with simvastatin, and GGPP (20 μmol/L) without or with simvastatin. Experiments were performed with at least three different HUVEC cultures and all data were compared to control, i.e. ethanol plus methanol. B. Shown are representative dot plots from a typical experiment. HUVEC incubated with vehicle (ethanol, 0.2% v/v; A, B, E, F), simvastatin (1.0 μmol/L; C, G) or simvastatin (5.0 μmol/L; D, H). Adherent (shown in panels A-D) and detached (panels E-H) cells were separately isolated and stained with FITC-labeled IgG control antibody (panels A and E) or anti-caspase 3 antibody (panels B-D and F-H).
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Figure 2A shows the increase in absolute numbers of detached cells containing caspase 3 in the presence of 1.0 or 5.0 μmol/L simvastatin (P=0.039 and P=0.041, respectively). The simvastatin-induced increases were completely blocked by co-incubation with mevalonate or GGPP. Since the fractions of both detached and adherent cells staining for caspase 3 were not affected by simvastatin (Figure 2B), these data may implicate that simvastatin may facilitate detachment rather than induce apoptosis. Figure 2C confirms the presence of caspase 3 in detached cell lysates from simvastatin-treated cultures (upper right). Since detached cells were isolated from a fixed volume of conditioned medium, the observed increase in caspase 3 is due to the increased number of detached cells. In contrast, in adherent cell lysates no caspase 3 could be detected (upper left). Co-incubation with either mevalonate or GGPP completely prevented the simvastatin-induced increase in caspase 3 formation (data not shown). Procaspase 3 was detectable only in adherent (lower left) but not in detached cells (lower right).

Since the presence of caspase 3 is no absolute evidence that cells are undergoing apoptosis, adherent and detached cells were stained for annexin V (early apoptosis) and PI (late apoptosis) in a control experiment from both control and 1.0 μmol/L simvastatin-treated cultures to determine whether or not simvastatin affects their apoptotic status (Figure 3). On average, only 3.8% ± 1.6 of adherent cells stained for annexin V and PI in this experiment, irrespective of the presence (Figure 3B and 3D) or absence (Figure 3A and 3C) of simvastatin. The total number of detached cells staining for annexin V or PI increased in the presence of simvastatin (Figure 3F and 3H versus 3E and 3G, respectively). The relative proportion of these (detached) cells staining for annexin V and PI, however, was unaffected by simvastatin (74% ± 10). Thus, simvastatin may not have a direct effect on the apoptotic status of adherent endothelial cells, and the occurrence of caspase 3 in this experiment closely parallels staining for annexin V and PI as markers of apoptosis.
Figure 2. A. Numbers of caspase 3-containing detached cells. B. Fractions of adherent (open bars) and detached (shaded bars) cells containing caspase 3. HUVEC were incubated (24 hours) without any additions (control), ethanol (0.2% v/v) plus methanol (0.2% v/v), Simvastatin (1.0 μmol/L and 5.0 μmol/L), mevalonate (100 μmol/L) without or with Simvastatin, and GGPP (20 μmol/L) without or with Simvastatin. Data are mean percentages of adherent cells and detached cells containing caspase 3. C. Western blot of caspase 3 and procaspase 3 in adherent and detached endothelial cell lysates from a single, representative experiment.
Figure 3. Effect of simvastatin on the apoptotic status of detached and adherent cells. HUVEC were incubated without (A, C, E, G) or with (B, D, F, H) simvastatin (1.0 μmol/L) for 24 hours. Adherent (A-D) and detached cells (E-H) were stained with annexin V (A, B, E, F) or PI (C, D, G, H).
Chapter 5

**Effect of simvastatin on EMP formation**

Upon incubation with either 1.0 or 5.0 μmol/L simvastatin, the numbers of caspase 3-containing EMP increased (Figure 4A; C and D, respectively) when compared to control (Figure 4A; A) or vehicle (Figure 4A; B). The numbers of annexin V-binding and caspase 3-containing EMP increased 2.5-fold in the presence of simvastatin (Figure 4B). This increase tended to be statistically significant at 1.0 μmol/L simvastatin and reached statistical significance at 5.0 μmol/L simvastatin (P=0.098 and P=0.041, respectively). Co-incubation with either mevalonate or GGPP completely abolished the statin-induced EMP release. The insert of Figure 4B confirms the presence of caspase 3 in EMP lysates. Both mevalonate and GGPP completely prevented the observed (simvastatin-induced) increase in caspase 3 formation (data not shown). Thus, prenylation counteracts the simvastatin-induced release of (caspase 3-containing) EMP.

**Role of caspases in statin-induced cell detachment**

Active caspase 3 (17 kDa) is a cleavage product of the inactive 32 kDa precursor (procaspase 3). Induction of programmed cell-death, either via death receptors (‘extrinsic’) or via leakage of mitochondrial cytochrome C (‘intrinsic’), ultimately leads to cleavage of procaspase 3 by either caspase 8 (‘extrinsic’) or caspase 9 (‘intrinsic’). Figure 5 shows that only procaspases 8 (57 kDa) and 9 (47 kDa) were detectable in adherent cell lysates, and their relative quantities seemed unaffected by simvastatin. In contrast, detached cells and to a lesser extent EMP contained detectable quantities of caspase 8 (43 kDa) after incubation with simvastatin, but not caspase 9 (35-37 kDa). Caspase 8 was not detectable when cells were co-incubated with mevalonate or GGPP (data not shown). Since caspase 8 but not caspase 9 is detectable in detached cell lysates from simvastatin-treated cultures, we hypothesize that cleavage of procaspase 8 may explain the observed increase in caspase 3 under these conditions.
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**Figure 4. A.** Flowcytometric histograms of caspase 3-containing EMP from a single, representative experiment. The light grey and black curves show IgG (control antibody) and (anti-) caspase 3, respectively. EMP were isolated from HUVEC conditioned media after 24 hours of incubation without addition (control; A), vehicle (0.2% v/v; B) or simvastatin (1.0 and 5.0 μmol/L; C and D, respectively). B. Absolute numbers of caspase 3-containing EMP. EMP were isolated from conditioned media as outlined in Methods. The insert shows the effect of simvastatin on the amounts of 17 kDa caspase 3 in EMP lysates.
Figure 5. (Pro-) Caspase 8 and 9 in adherent cells, detached cells and EMP of HUVEC incubated (24 hours) without any additions (control), ethanol (0.2% v/v) plus methanol (0.2% v/v), simvastatin (1.0 μmol/L) or simvastatin (5.0 μmol/L). Shown are representative Western blots from a typical experiment.

DISCUSSION

Incubation of human endothelial cells with simvastatin at clinically relevant doses triggered endothelial cell detachment as well as EMP release. Lysates from both detached cells and EMP contained substantial quantities of caspases 3 and 8, whereas caspase 9 remained below the detection limit, suggesting that caspase 8 formation underlies the formation of caspase 3 under these conditions. Previously, statins were shown to induce apoptosis of keratinocytes via ligand-independent activation of caspase 8 via a death receptor [23]. Whether or not formation of caspase 8 precedes detachment or is a consequence of detachment (‘anoikis’), however, remains to be determined.

Caspase 3 cleaves focal-adhesion kinase, thus eliminating essential cellular survival signals and thereby facilitating detachment. In addition, caspase 3 cleaves kinases like Rho-associated coiled kinase (ROCK)-I and p21-kinase, resulting in the formation of constitutively active kinases which directly contribute to formation of “apoptotic bodies” [24,25]. Therefore, we hypothesize that -similar to keratinocytes- simvastatin may trigger caspase 8-mediated endothelial detachment and EMP release.
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Our present data indicate that simvastatin-induced detachment and EMP release can be circumvented by restoring prenylation. Also, other investigators showed that statins induce apoptosis of endothelial cells in vitro, and in most studies these effects were counteracted by restoring prenylation. The extent by which statins induce apoptosis, however, seems to be dependent on the type of endothelial cell studied. With particular regard to HUVEC, however, statins induce a wide variety of effects, including enhanced expression of tissue factor and adhesion receptors, an increased release of EMP and augmented production and bioavailability of endothelium-derived NO [10,12,13,16,26-30]. In some of these studies, data are difficult to interpret since pharmacological and potentially cytotoxic concentrations of statins were used or statins were used only in combination with other endothelial agonists like TNF-α or endotoxin. In most of these studies, however, solely adherent endothelial cells were studied. Our present data suggest that to appreciate the full scope of the statin effects on endothelial cells, all individual components of the incubation well, i.e. adherent cells, detached cells and MP, should be taken into account. In this regard, it should be mentioned that in some studies the apoptotic effects of statins on (adherent) endothelial cells could only be observed in the presence of additional inducers like TNF-α, whereas in the present study the pro-apoptotic effect of statin-treatment alone became apparent when not only adherent cells but also detached cells and EMP were analyzed.

Whereas some potentially harmful adverse effects of statins have been reported on endothelial cells in vitro, these drugs have many beneficial effects on the endothelium in vivo. Statins increase the number and survival of circulating endothelial progenitor cells, facilitate re-endothelialization, inhibit endothelial senescence and increase cell proliferation by affecting cell cycle genes [31]. Thus, in spite of these seemingly contradictory reports, the existence of a discrepancy between in vitro versus in vivo effects of statins may be questioned. In our present study, we showed that adherent endothelial cells seemed unaffected by treatment with simvastatin despite increased numbers of both 'floaters' and EMP. Possibly, by facilitating detachment and EMP release, statins may improve the overall condition of the vascular endothelium.

Previously, we reported a strong correlation between the numbers of detached endothelial cells and EMP [20]. Also, our present data indicate a close association
between cell detachment and EMP release. From such data, however, we may not conclude that EMP are released from detached cells since other investigators showed that EMP are released from still adherent cells during the process of detachment [24,32].

In summary, based on the present data we hypothesize that statins may facilitate cell detachment and EMP release in order to preserve the overall condition and anti-atherogenic properties of the vascular endothelium. We suggest that, in order to gain full insight into the effects of compounds on endothelial cell biology, evaluation of adherent cells, detached cells as well as EMP should be adopted as a general methodological principle.
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References


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