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

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Summary

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 functioning *in vitro* and *in vivo*. We investigated whether simvastatin affects endothelial detachment and release of EMP. Human umbilical vein endothelial cells (HUVECs) were incubated with clinically relevant concentrations of simvastatin (1.0 and 5.0 μM), with or without mevalonic acid (100 μM) or geranylgeranylpyrophosphate (GGPP; 20 μM) for 24 hours, and analyzed by flowcytometry and Western blot. Simvastatin at 1.0 and 5.0 μM increased cell detachment from 12.5 ± 4.1% to 26.0 ± 7.6% (p=0.013) and 28.9 ± 2.2% (p=0.002) as well as EMP release (p=0.098 and p=0.041, respectively). The majority of detached cells was apoptotic, although the fraction of detached cells that showed signs of apoptosis (>70%) was unaffected by simvastatin. Detached cells and EMP contained caspase 3 and caspase 8, but not caspase 9. Restoring either cholesterol biosynthesis and prenylation (mevalonate) or prenylation alone (GGPP) reversed all simvastatin-induced effects on cell detachment and EMP release. Adherent cells showed no signs of simvastatin-induced apoptosis. 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 improve the overall condition of the remaining vascular endothelium.

Keywords

Anoikis, apoptosis, cardiovascular disease, endothelium, microparticles, statins

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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-C). Their beneficial effects in cardiovascular disease (CVD) patients have been largely attributed to their efficacy to lower LDL-C (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-C 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, are associated with cell membranes and are essential for transmembrane signaling (19). As a result, G proteins are involved in the

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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 apoptosis as well as caspase 3-containing MP (EMP) (20). Although we and others reported a strong positive correlation between EMP and detached cells, suggesting that (most) EMP become released during or immediately after detachment, we recently showed that caspase 3 accumulates in adherent endothelial cells in the presence of inhibitors of MP release. This accumulation results in apoptosis and detachment, suggesting that the release of caspase 3-containing EMP contributes to endothelial survival (21).

Since in the aforementioned studies on the effects of statins on endothelial cells *in vitro* little attention was paid to detachment and/or release of EMP, we hypothesized that in order to study the full impact of statins on the human endothelium, not only the adherent endothelial cell fraction but also the corresponding fractions of detached endothelial cells and EMP have to be studied.

Materials and methods

Reagents and assays

Medium M199, penicillin, streptomycin, Iscove's modified dulbecco's medium and L-glutamin were obtained from GibcoBRL, Life Technologies (Paisley, Scotland, UK). Human serum and fetal calf serum (both heat inactivated for 30 minutes (min) at 56°C; HuSi and FCSi, respectively) were from BioWhittaker (Walkersville, MD, USA). 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, USA). Collagenase (type 1A), geranylgeranylpyrophosphate (GGPP) and mevalonolactone (mevalonate) were from Sigma (St. Louis, MO, USA). Propidium iodide (PI) was a gift from Dr. E. Reits (Department of Cell Biology of the AMC, Amsterdam, The Netherlands). Allophycocyanin (APC)-labeled annexin V was from Caltag Laboratories (Burlingame, CA, USA). Heparin (400 U/ml) was from Leo Pharma BV (Breda, The Netherlands), trypsin from Difco Laboratories (Detroit, MI, USA), and simvastatin from Calbiochem (Darmstadt, Germany). Tissue culture flasks were from Greiner Labortechnik (Frickenhausen, Germany) and gelatin from Difco Laboratories (Sparks, MD, USA). Stock solutions of simvastatin, mevalonate and GGPP were prepared in ethanol, and 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, USA). Anti-procaspase 3 was from Transduction Laboratories (San Diego, CA, USA). Secondary antibodies used for Western blot, i.e. goat-anti-mouse HRP conjugate and anti-rabbit HRP conjugate, were from Biorad (Hercules, CA, USA) and Promega (Madison, WI, 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 previously described (20). Briefly, umbilical cords were digested with collagenase for 20 min 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 min at 180 x *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 three to four days in a resting state. Then, the culture supernatant was refreshed and (where indicated) cultures were treated for 24 hours (h) 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 (22, 23).

Flow cytometric analysis of endothelial cells

Conditioned media (10 ml per 75 cm^2 flasks) were harvested after 24 h. First, media were centrifuged for 10 min at 180 x *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 min, trypsin was neutralized by PBS/FCSi. Both cell suspensions were separately centrifuged for 10 min at 180 x *g* and 4 °C, resuspended in PBS/FCSi, kept on melting ice for 15 min, and then again centrifuged for 10 min at 180 x *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 Mab Apoptosis Kit I was used (BD Pharmingen; San Diego, CA, USA). From the previously 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 min at 180 x *g*. After removal of the supernatant, the cells were again diluted with 1 ml of ice-cold PBS and pelleted (10 min at 180 x *g*). After removal of the supernatant, cells were resuspended in 500 μl cytofix/cytoperm and incubated for 20 min on melting ice. To remove the cytofix/cytoperm, cells were pelleted (10 min at 180 x *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 min 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 min at 180 x *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). Forward scatter and side scatter were set at linear scale. Percentages of adherent and detached cells were compared to the total cell count (i.e. adherent plus detached cells)/culture flask (100%).

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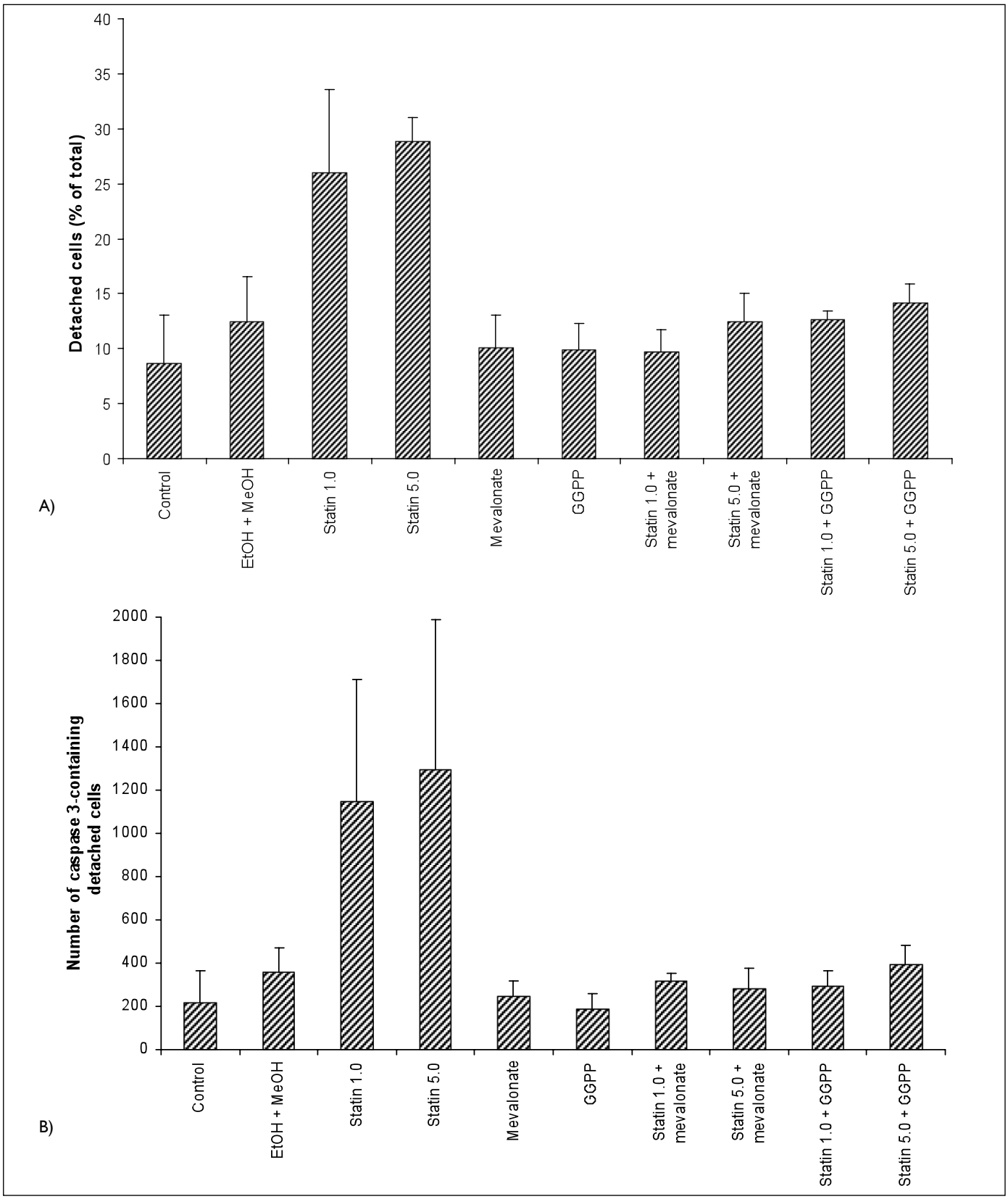

Figure 1: Simvastatin induces endothelial detachment. A) Simvastatin induces endothelial detachment. HUVEC were incubated (24 hours) without any additions (control), ethanol (0.2% v/v) plus methanol (0.2% v/v), simvastatin (1.0 μM and 5.0 μM), mevalonate (100 μM) without or with simvastatin, and GGPP (20 μM) with or without simvastatin. Experiments were performed with at least three different HUVEC cultures and all data were compared to control, i.e. ethanol plus methanol. B) Number of caspase 3-containing detached cells. C) Western blot of caspase 3 and procaspase 3 in adherent and detached endothelial cell lysates from a single, representative experiment.

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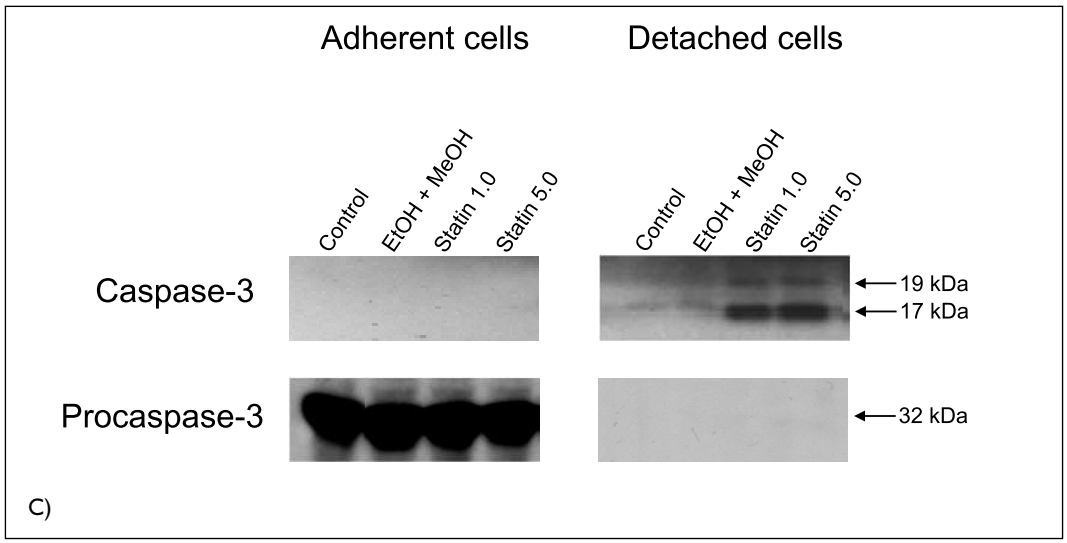

Figure 1 continued

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 one hour, and then centrifuged for 1 h at 17,570 x *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 flowcytometry and the other aliquot for intravesicular caspase 3 staining.

For regular flow cytometry, 25 µl EMP suspension was diluted with 225 µl PBS (154 mM NaCl, 1.4 mM phosphate) containing 10.9 mM trisodium citrate. EMP were resuspended and again centrifuged for 30 min at 17,570 x *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 min at 17,570 x *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). Forward scatter (FSC) and side scatter (SSC) were set at logarithmic gain and EMP were characterized as previously described by binding of annexin V. EMP (5 µl aliquots) were diluted with 45 µl PBS containing 2.5 mM $CaCl_2$ (pH 7.4). APC-labeled annexin V (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 Ca^{2+}. The mixture of MP and annexin V was then incubated for 15 min 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 min 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, detached cells and adherent cells were isolated by centrifugation and diluted with five-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 five-fold concentrated sample buffer. Before electrophoresis, all samples were heated for five min at 100°C. Electrophoresis was carried out on 8–16% gradient polyacrylamide gel (Biorad, Hercules, CA, USA). The proteins were transferred to PVDF membrane (Biorad). Blots were incubated for 1 h at room temperature with blocking buffer (Tris-buffered saline-Tween (TBST); 10 mM Tris-HCl, 150 mM 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) overnight 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 one hour at room temperature. After each incubation step, PVDF membranes were washed three times with TBST for five to 10 min. 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, USA). Differences were analyzed by t-test for independent samples and were considered to be significant at

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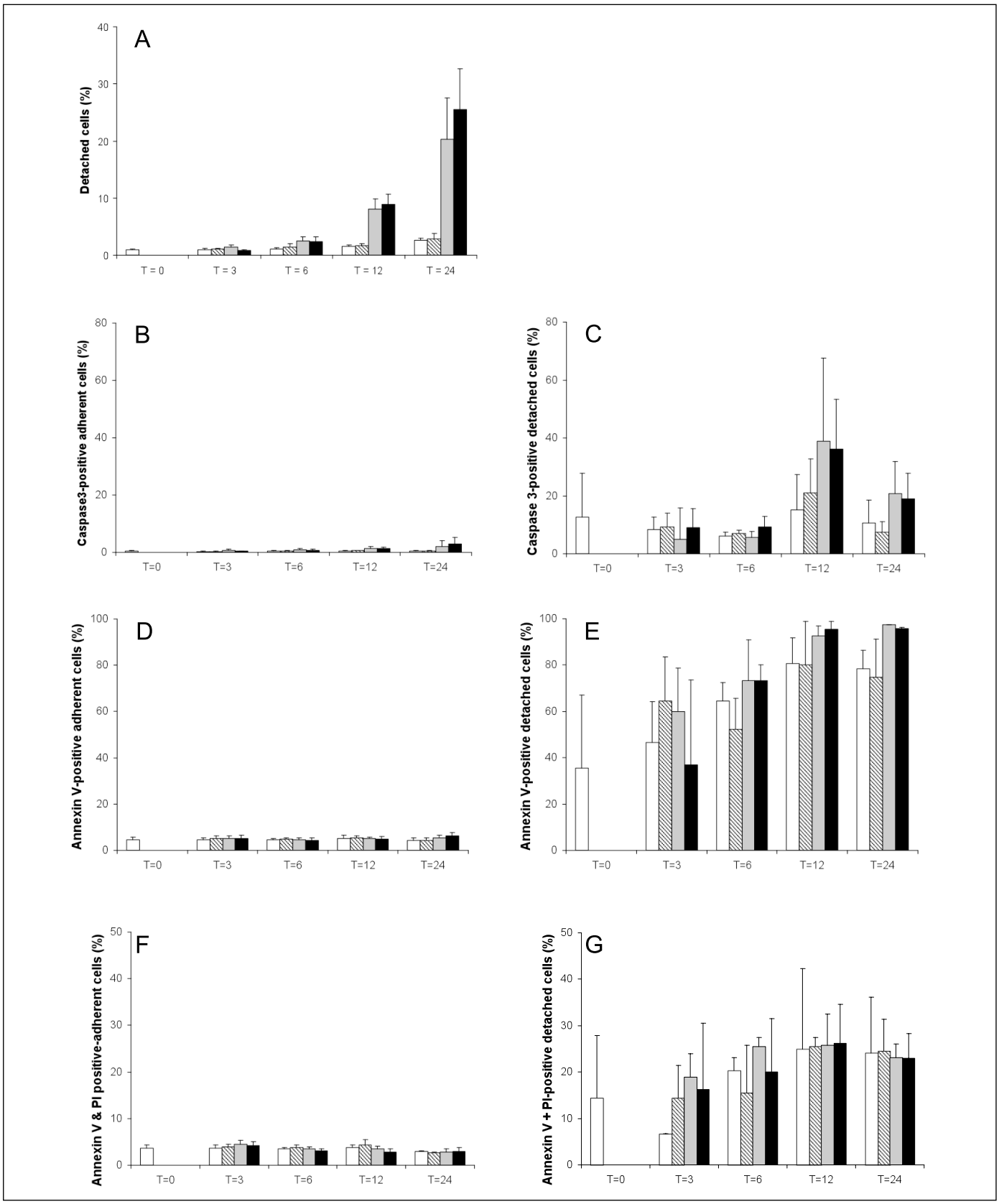

Figure 2: Effect of simvastatin on detachment and the apoptotic status of adherent and detached endothelial cells. HUVEC were incubated without any additions (control; open bars), ethanol (0.2% v/v; striped bars), or simvastatin (1.0 μM and 5.0 μM; grey and black bars, respectively). The intracellular staining for caspase 3 (B, C), binding of annexin V (D, E) or binding of annexin V and staining for PI (F, G) were studied in both adherent (B, D, F) and detached endothelial cell fractions (C, E, G) at the indicated time intervals (n=3).

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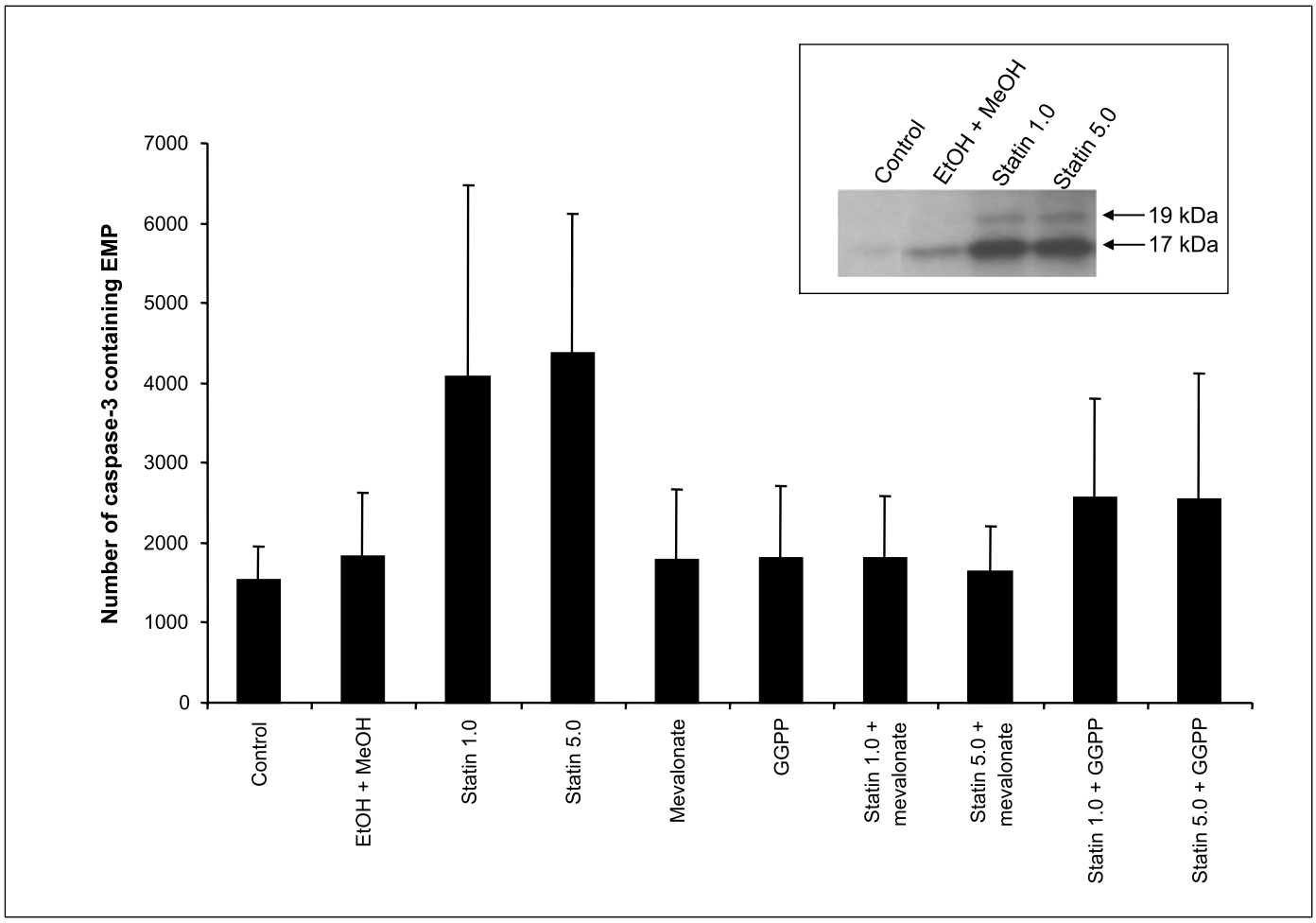

Figure 3: Absolute numbers of caspase 3-containing EMP. EMP were isolated from conditioned media as outlined in *Materials and methods*. The insert shows the effect of simvastatin on the amounts of 17 kDa caspase 3 in EMP lysates.

$p<0.05$. With regard to the time-dependent effects of simvastatin, area under curves were calculated and differences were analyzed using (two-tailed) Mann-Whitney test (MedCalc). All data are presented as mean ± SD. Data were obtained from at least three independent experiments, i.e. using endothelial cell cultures from three or more different umbilical veins. Data were compared to endothelial cells incubated with ethanol- (EtOH) plus methanol (MeOH).

Results

Simvastatin induces endothelial cell detachment

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

Figure 1B shows the increase in absolute numbers of detached cells containing caspase 3 in the presence of 1.0 or 5.0 μM 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 (data not shown), these data imply that simvastatin enhances detachment rather than induces apoptosis.

The presence of caspase 3 in detached cell lysates from simvastin-treated cultures was confirmed by Western blot (Fig. 1C, upper right blot). In contrast, in adherent cell lysates no caspase 3 could be detected (upper left blot). 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 but not in detached cells (lower left and lower right blot, respectively).

Simvastatin induces endothelial detachment but not apoptosis

Because the presence of caspase 3 (Fig. 1B) is not absolute proof of a cell undergoing apoptosis, and in order to gain further insight into the complex relationship between detachment and

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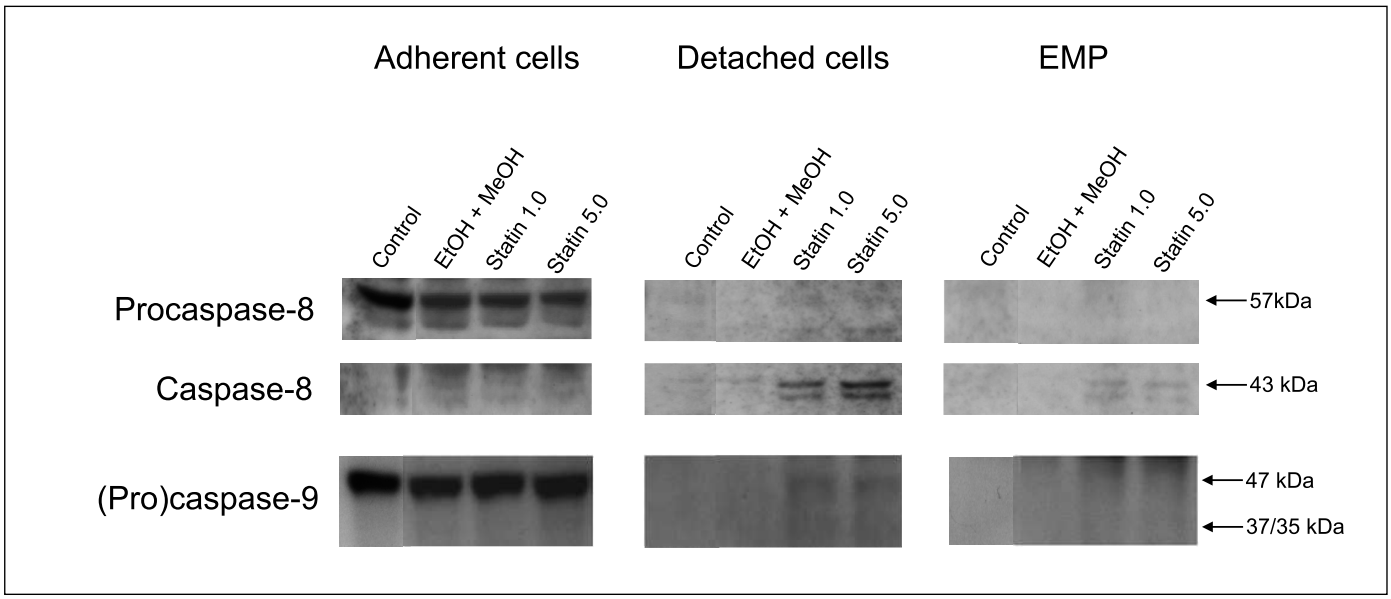

Figure 4: (Pro-) Caspase 8 and 9 in adherent cells, detached cells and EMP. HUVEC were incubated (24 h) without any additions (control), ethanol (0.2% v/v) plus methanol (0.2% v/v), simvastatin (1.0 μM) or simvastatin (5.0 μM). Representative Western blots from a typical experiment are shown.

apoptosis, we also studied the effects of simvastatin on detachment and apoptosis in time (0–24 h). Figure 2A shows that detachment increased significantly during incubation with simvastatin 1.0 or 5.0 μM (both $p<0.0001$). With regard to the apoptosis status of the adherent cells, no increases were observed in the fractions staining for caspase 3 (Fig. 2B), or those staining for annexin V (Fig. 2D) or annexin V plus PI (Fig. 2F). With regard to detached cells, fractions staining for caspase 3, annexin V or annexin V plus PI increased up to 12 hours (Figs. 2C, E and G, respectively). The size of these fractions, however, were unaffected by simvastatin. In conclusion, simvastatin increases endothelial detachment without concurrent signs of adherent cells undergoing apoptosis. Also, the fraction of apoptotic, detached endothelial cells was not affected by simvastatin.

Effect of simvastatin on EMP formation

The numbers of annexin V-binding and caspase 3-containing EMP as measured by flow cytometry increased in the presence of simvastatin (Fig. 3). This increase tended to be statistically significant at 1.0 μM simvastatin and reached statistical significance at 5.0 μM simvastatin ($p=0.098$ and $p=0.041$, respectively). Co-incubation with either mevalonate or GGPP almost completely reversed the statin-induced EMP release. Thus, prenylation counteracts the simvastatin-induced release of (caspase 3-containing) EMP.

The insert of Figure 3 confirms the presence of caspase 3 in EMP lysates using Western blot analysis.

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 or caspase 9. Figure 4 shows that procaspases 8 (57 kDa) and 9 (47 kDa) were detectable in adherent cell lysates, and their relative quantities seemed unaffected by simvastatin. The active forms of both enzymes, however, were not detectable in adherent cells. 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). Since detached cell lysates from simvastatin-treated cultures contained caspase 8 but not caspase 9, these data indicate that procaspase 3 is cleaved by caspase 8 under these conditions.

Discussion

Incubation of human endothelial cells with simvastatin at clinically relevant doses triggered cell detachment and EMP release, whereas adherent endothelial cells remained viable and showed no signs of apoptosis.

Lysates from detached cells and EMP contained substantial quantities of caspases 3 and 8, whereas caspase 9 remained below the detection limit, suggesting that caspase 8 mediates the formation caspase 3 under these conditions. Previously, statins were shown to induce apoptosis of keratinocytes via ligand-independent activation of caspase 8 by a death receptor (24). To which extent formation of caspase 8 precedes detachment or is a consequence of detachment ('anoikis') in our present experiments remains to be determined. Because we did not observe any increases in the numbers of adherent cells containing caspase 3, binding annexin V (early apoptosis), or staining for annexin V plus PI (late apoptosis) during incubation with simvastatin, our data indicate that detachment precedes apoptosis. Caspase 3 cleaves focal-adhesion kinase, thus eliminating essential cellular survival signals and thereby facilitating detachment. In addition,

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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 the formation of "apoptotic bodies" (25, 26). Therefore, we hypothesize that, similar to keratinocytes, simvastatin triggers caspase 8-mediated activation of caspase 3, with associated cell detachment and EMP release.

Our present data indicate that simvastatin-induced detachment and EMP release can be circumvented by restoring prenylation. Other investigators also 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, 27–31). In some of these studies the 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 studies, however, solely adherent endothelial cells were studied. Our present data suggest that in order 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.

The current view on the role of MP is changing. Already in 2003, Freyssinet proposed that MP may not only impose a potential environmental threat, but may also be essential to maintain (cellular) homeostasis (32). Ever since, the latter has been supported by several studies from ourselves and others. Thus, the release of MP protects cells against complement-induced lysis, and against the accumulation of caspase 3 or anti-cancer drugs (21, 33–35). In addition, although the occurrence of MP *in vivo* has often been associated with an increased cardiovascular risk (36, 37), it should be stressed that MP also have beneficial properties. Accordingly, MP were shown to have anticoagulant properties, to induce a pro-inflammatory response which is regarded as an essential protective mechanism contributing to the organisms' survival, and to facilitate fibrin formation and to promote wound healing by delivering the initiator of coagulation to the platelet surface (38–44). Thus, elevated numbers of EMP in patients with diabetes or CVD, relative to healthy controls, are likely to reflect an activated cellular mechanism to cope with increased stress or the endothelial homeostasis maintenance in overdrive, rather than a mechanism to impose an environmental threat to other cells and tissues. This finding also implicates that the endothelium of these high-risk patients is either more susceptible or exposed to more stress than healthy endothelium. That EMP indeed reflect "homeostasis" is confirmed in recent studies, which invariably reported a relationship between endothelial (dys)function *in vivo*, such as flow-mediated vasodilation, and circulating EMP (45, 46).

Further evidence to demonstarte that MP-release may be a beneficial adaptive mechanism was obtained from experiments showing that inhibition of EMP-release triggered accumulation of caspase 3 in endothelial cells, which was paralleled by apoptosis and detachment (21). Based on these observations we hypothesize that the statin-related increased EMP-release as observed in the present study may improve the overall ability of the endothelium to cope with stress, in this way contributing to the management of CVD. To which extent statins contribute to the release of EMP *in vivo* is unknown, but the fact that many patients with CVD and diabetes who were reported to have elevated levels of EMP were using statins, points to a possible similar role *in vivo*. Prospective clinical studies in well-defined patient populations who have CVD prior to and after statin treatment may resolve those issues.

In summary, based on the present data we hypothesize that statins facilitate cell detachment and EMP release in order to preserve the overall condition and anti-atherogenic properties of the remaining 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 to fully appreciate the effect of statins on endothelial cells.

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