Cerivastatin, an Inhibitor of 3-Hydroxy-3-Methylglutaryl Coenzyme A Reductase, Inhibits Endothelial Cell Proliferation Induced by Angiogenic Factors In Vitro and Angiogenesis in In Vivo Models

Loïc Vincent, Claudine Soria, Farrokh Mirshahi, Paule Opolon, Zohair Mishal, Jean-Pierre Vannier, Jeannette Soria, Li Hong

Abstract—Cerivastatin is an inhibitor of 3-hydroxy-3-methylglutaryl coenzyme A reductase. It inhibits the biosynthesis of cholesterol and its precursors: farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), which are involved in Ras and RhoA cell signaling, respectively. Statins induce greater protection against vascular risk than that expected by cholesterol reduction. Therefore, cerivastatin could protect plaque against rupture, an important cause of ischemic events. In this study, the effect of cerivastatin was tested on angiogenesis because it participates in plaque progression and plaque destabilization. Cerivastatin inhibits in vitro the microvascular endothelial cell proliferation induced by growth factors, whereas it has no effect on unstimulated cells. This growth arrest occurs at the G1/S phase and is related to the increase of the cyclin-dependent kinase inhibitor p21WAF1/CIP1. These effects are reversed by GGPP, suggesting that the inhibitory effect of cerivastatin is related to RhoA inactivation. This mechanism was confirmed by RhoA delocalization from cell membrane to cytoplasm and actin fiber depolymerization, which are also prevented by GGPP. It was also shown that RhoA-dependent inhibition of cell proliferation is mediated by the inhibition of focal adhesion kinase and Akt activations. Moreover, cerivastatin inhibits in vivo angiogenesis in matrigel and chick choioallantoic membrane models. These results demonstrate the antiangiogenic activity of statins and suggest that it may contribute to their therapeutic benefits in the progression and acute manifestations of atherosclerosis. (Arterioscler Thromb Vasc Biol. 2002;22:623-629.)

Key Words: statins ■ angiogenesis inhibition ■ RhoA ■ vascular risk

Neovascularization is an important process that is required for the progression of atherosclerosis.1,2 Increased vascularization is recognized as a major feature during plaque development.3 Its pathogenic role includes the supply of nutrients and oxygen and the accumulation of blood monocytes, which pass through the endothelium.4 In addition, angiogenesis could also be involved in destabilization of the plaque, leading to its rupture, which causes thrombosis and acute ischemic events.5 Moreover, the role of angiogenesis in atherosclerosis has been demonstrated by the histochemical examination of atherosclerotic plaque from autopsy specimens by Boyle et al,6 who showed a strong correlation between the severity of stenosis and plaque microvascular density. In addition, by examining plaque from patients with ischemic events, Jeziorska and Woolley7 reported that local hemorrhages derived from microvessels within the atherosclerotic plaque contribute to the acute complications of atherosclerosis by favoring plaque destabilization. McCarthy et al8 also reported that there were significantly more neovessels in plaques and fibrous caps in symptomatic compared with asymptomatic plaques.

Possible stimuli for plaque angiogenesis include local hypoxia and leukocyte-derived cytokines, such as vascular endothelial growth factor (VEGF), which has been detected in atherosclerotic plaque, or basic fibroblast growth factor (bFGF).9 Oncostatin M (OSM), secreted by activated monocytes and which is largely overexpressed in atherosclerotic plaque (especially in areas of aneurysm),10 has also recently been characterized as an angiogenic factor.11

Because the beneficial effect of inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (these inhibitors are commonly named statins) in the prevention of vascular risk is extended beyond the lowering of plasma cholesterol levels,12 we were prompted to analyze the effect...
of a statin, cerivastatin, on endothelial cell proliferation and angiogenesis. Statins lead to a decreased synthesis of cholesterol as well as its precursors, which are isoprenoid products of mevalonate (MVA). The inhibition in the formation of these isoprenoids, farnesyl pyrophosphate (FPP) and geranylgeranyl pyrophosphate (GGPP), could play an important role in altering the processing of signaling that requires lipophilic anchors to cell membranes. FPP is essential for membrane attachment and biological activity of small GTP-binding proteins from the Ras family,13 and GGPP is required for RhoA translocation to cell membranes.14

In the present study, we have shown that cerivastatin repressed in vitro the proliferative activity of angiogenic factors on microvascular endothelial cells (human microvascular endothelial cells [HMEC-1 cells]), which are representative of atherosclerotic plaque vascularization. Moreover, cerivastatin inhibits angiogenesis in vivo in the matrigel model in mice and in the chick chorioallantoic membrane (CAM).

These observations raised the important question of the mechanism by which cerivastatin induces its antiangiogenic effect. In the present study, we first examined the relative participation of RhoA and Ras inhibition on the antiangiogenic effect of cerivastatin. Second, we analyzed the effect of cerivastatin on cell cycle distribution and on the expression of the cyclin-dependent kinase inhibitor p21Waf1/Cip1, a negative regulator of cell cycle progression in late G1 phase. Third, to understand the molecular mechanism involved in this inhibition, we evaluated the modification of cell signaling induced by cerivastatin on bFGF-stimulated endothelial cells. As we reported that cerivastatin inhibits the formation of focal adhesion sites where focal adhesion kinase (FAK) is activated in the presence of Rho, we were prompted to particularly analyze the effect of cerivastatin on the cell-signaling cascade FAK/phosphatidylinositol (PI)-3-kinase (PI3K)/Akt. This was justified by the fact that phosphorylated Akt is markedly implicated in endothelial cell survival, proliferation, and angiogenesis.16–18

Methods

Please see online data supplement (which can be accessed at http://www.atvb.ahajournals.org) for materials and methods concerning cytokines and cerivastatin, cell culture, cell proliferation assay, apoptosis analysis, adhesion assay, determination of cell cycle analysis, Western blot analysis, RNA isolation, reverse transcriptase–polymerase chain reaction assay, ELISA of p21Waf1/Cip1, confocal microscopy analysis of RhoA and actin filaments, in vivo angiogenesis assays (matrigel model and chick CAM model), and statistical analysis.

Results

Cerivastatin Inhibits Endothelial Cell Proliferation

From 10 to 25 ng/mL, cerivastatin induced a dose-dependent decrease in stimulated–endothelial cell proliferation in the presence of 2.5 ng/mL OSM, 25 ng/mL bFGF, or 20 ng/mL VEGF (Figure 1A and 1B). This phenomenon was associated with cell rounding. Higher concentrations were not tested because 50 ng/mL cerivastatin induced cell detachment from the surface of the well and, consequently, cell death, as assessed by the incorporation of trypan blue. In contrast, cerivastatin did not change the proliferation of unstimulated endothelial cells.

To analyze the contribution of isoprenoid synthesis inhibition by cerivastatin on its antiproliferative effect, we tested whether this inhibitory effect could be reversed by 100 μmol/L MVA, 10 μmol/L GGPP, or 10 μmol/L FPP. MVA and GGPP totally prevented the inhibitory effect of cerivastatin (10 and 25 ng/mL) on cytokine-induced endothelial cell proliferation (Figure 2A through 2D), whereas FPP did not prevent the inhibitory effect of cerivastatin (data not shown). These results suggest that cerivastatin induced an antiproliferative effect by the blockage of GGPP formation.

Cerivastatin Does Not Induce Endothelial Cell Apoptosis

Analysis of apoptosis by Hoechst 33342 staining was performed on bFGF-stimulated endothelial cells in the presence
or absence of cerivastatin (10 and 25 ng/mL). Results of 4 individual experiments showed that a 24-hour incubation of endothelial cells with cerivastatin did not modify the apoptosis measured on adherent cells: the ratio of the number of apoptotic cells in cerivastatin-treated cells to the number of apoptotic cells in control cells was 1.07/0.06 and 0.97/0.04 for 10 ng/mL and 25 ng/mL cerivastatin, respectively. Moreover, PI incorporation was not observed, confirming the absence of cell permeability (data not shown).

Cerivastatin Induces Endothelial Cell Detachment
The effect of cerivastatin on endothelial cell detachment was expressed as the percentage of detached endothelial cells cultured in the presence of cerivastatin compared with that of control cells. Cerivastatin (25 ng/mL) induced a mild but significant increase in detached endothelial cells whether or not the cells were stimulated by bFGF (155±15% and 142±11% of control cells for cells stimulated with and without bFGF, respectively; P<0.001; n=3).

Cerivastatin Blocks G1/S Transition of Cell Cycle
Treatment with 10 or 25 ng/mL cerivastatin in the presence or absence of stimulation by OSM for 18 hours resulted in the accumulation of cells in the G0/G1 phase (online Figure IA; please see http://www.atvb.ahajournals.org), with a corresponding decrease in the number of cells in G2/M phase (online Figure IC). Cerivastatin treatment led to a significant reduction in OSM-stimulated endothelial cells in S-phase cells (online Figure IB). These results clearly demonstrate that cerivastatin controls cell cycle progression by an inhibition of the cell cycle at the G1 phase. Moreover, the effect of cerivastatin on G1-phase cell cycle blockage was fully reversible by coincubation with MVA or GGPP but not with FPP (data not shown).

Cerivastatin Increases mRNA and Protein Expression of the Cyclin-Dependent Kinase Inhibitor p21Waf1/Cip1
In HMEC-1 cells, we assessed whether cerivastatin induced p21Waf1/Cip1, an inhibitor of the G1-phase cell cycle. As shown in Figure 3, cerivastatin (10 and 25 ng/mL) induced, in a dose-dependent manner, a marked increase in the level of p21Waf1/Cip1 protein and mRNA on unstimulated and bFGF-stimulated endothelial cells. To investigate whether RhoA or Ras inactivation accounted for the induction of p21Waf1/Cip1, we analyzed the effect of FPP and GGPP by ELISA. When HMEC-1 cells were treated with cerivastatin together with GGPP (10 μmol/L) or FPP (10 μmol/L), only GGPP reversed the stimulating effect of cerivastatin on p21Waf1/Cip1 expression (Table).

Cerivastatin Decreases the Phosphorylations of FAK (Phospho-Tyr397) and Akt (Phospho-Ser473)
We studied the effect of cerivastatin on FAK phosphorylation (Tyr397), which is mainly involved in the activation
of the PI3K/Akt pathway. The incubation of bFGF-stimulated endothelial cells with cerivastatin induced a decrease in the phosphorylated FAK from 12 hours of incubation with 25 ng/mL cerivastatin. During the same period, a decrease in the phosphorylated Akt (Ser473) was induced by cerivastatin treatment and was maintained at 24 hours. These reductions are clearly related to a decrease in phosphorylation, because the total amount of FAK and Akt was not modified by cerivastatin treatment (Figure 4).

Cerivastatin-Induced Delocalization of RhoA From Cell Membrane and Actin Depolymerization Is Reversed by GGPP
As described in our previous study, 15 in the absence of cerivastatin, RhoA was present at the membrane periphery and at the lamellipodia extensions of bFGF-treated cells. After a 24-hour treatment with 25 ng/mL cerivastatin, a rounding of the cells was observed with the disappearance of the lamellipodia. In addition, RhoA was translocated in the cytoplasm, mainly in the perinuclear region, and this effect led to the disruption of actin stress fibers. 15 As shown in online Figure II (please see http://www.atvb.ahajournals.org), the effect of cerivastatin on RhoA delocalization and actin depolymerization was completely reversed by coincubation with GGPP (10 μmol/L). We verified that in the absence of the first antibody, no fluorescence was detected as control (data not shown).

Cerivastatin Inhibits bFGF-Induced Angiogenesis in Matrigel Model
In this model, subcutaneously injected matrigel rapidly solidified at body temperature, thereby trapping bFGF and cerivastatin. Mice that received matrigel containing 1 μg bFGF and 100 μg cerivastatin developed a limited number of discontinuous vessel spots around the matrigel plugs (data not shown).

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### Table: Effect of FPP and GGPP on Cerivastatin-Induced Increase of p21\(^{Waf1/Cip1}\) Level by ELISA Assay

<table>
<thead>
<tr>
<th></th>
<th>Without Cerivastatin</th>
<th>Cerivastatin 25 ng/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1</td>
<td>3.08±0.12*</td>
</tr>
<tr>
<td>OSM</td>
<td>1.20±0.11</td>
<td>4.31±0.20*</td>
</tr>
<tr>
<td>FPP</td>
<td>0.96±0.18</td>
<td>4.17±0.18*</td>
</tr>
<tr>
<td>OSM+FPP</td>
<td>1.12±0.09</td>
<td>3.82±0.23*</td>
</tr>
<tr>
<td>GGPP</td>
<td>1.04±0.11</td>
<td>1.27±0.13</td>
</tr>
<tr>
<td>OSM+GGPP</td>
<td>0.86±0.15</td>
<td>1.12±0.17</td>
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Results (mean±SEM) are the average of 4 independent experiments (*P<0.001 as compared with untreated cells; n=4). OSM, 2.5 ng/mL; FPP, 10 μmol/L; GGPP, 10 μmol/L.

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### Figure 3. Effect of cerivastatin on p21\(^{Waf1/Cip1}\) mRNA and protein expression in HMEC-1 cells in presence or absence of bFGF. Cell extracts of HMEC-1 cells were analyzed by Western blot with the use of a monoclonal anti-p21\(^{Waf1/Cip1}\) antibody. Blots were developed with an enhanced chemiluminescence (ECL) reagent. Analysis of p21\(^{Waf1/Cip1}\) and β-actin mRNA expression was assessed by reverse transcriptase–polymerase chain reaction.
Angiogenesis in the Chick CAM Is Inhibited by Cerivastatin

The antiangiogenic effect of cerivastatin in vivo was also evaluated in the chick CAM. Chick embryos were incubated with PBS (for control) or cerivastatin (50 ng) at day 6 of embryonic development with the use of an established shellless culture technique. The effect of the additions on CAM angiogenesis was then analyzed after 4 days of incubation. Cerivastatin led to an efficient inhibition of angiogenesis in chick CAM. CAMs of 6-day-old chick embryos were incubated with PBS or cerivastatin (50 ng) for 4 days. Numerous vessels are seen when CAM is incubated with PBS. When the CAM is incubated with cerivastatin, the formation of new vessels is markedly decreased compared with control. Photographs of CAM are representative of each group of treated CAM. Arrows indicate blood vessels. Bar=900 μm.

Because statins, by inhibiting HMG-CoA reductase, prevent the synthesis of isoprenoid intermediates in the cholesterol biosynthetic pathway (FPP and GGPP), they may have pleiotropic effects on vascular wall cells. Moreover, FPP and GGPP are involved in membrane localization and cell signaling of the small GTP-binding proteins Ras and RhoA, respectively. Therefore, by preventing FPP and GGPP formation, statins could inhibit cellular events related to Ras or RhoA signaling.

Because angiogenesis plays a major role in the development and instability of atherosclerotic plaque, we sought to determine the effect of a statin, cerivastatin, in endothelial cell proliferation in vitro and angiogenesis in vivo. In our previous work, we have demonstrated that by inhibiting RhoA cell signaling, cerivastatin strongly inhibits endothelial cell locomotion and the matrix metalloproteinase-2 secretion involved in cell invasion and capillary tube formation. This effect was dependent on RhoA inhibition. However, cerivastatin does not modify the expression of urokinase by endothelial cells.

In the present study, we showed that cerivastatin inhibits, in a dose-dependent manner, the proliferation of microvascular endothelial cells when stimulated by angiogenic factors, such as bFGF, VEGF, and OSM, without an effect on apoptosis. Interestingly, cerivastatin did not modify the proliferation of endothelial cells in the absence of angiogenic factors. The antiproliferative effect of cerivastatin was fully reversible by coincubation with MVA or GGPP but not with FPP. This indicates that the inhibitory effect of cerivastatin on endothelial cell proliferation is mainly due to the inhibition of RhoA geranylization and not Ras farnesylation. Consistent with this observation, it was shown that cerivastatin induces actin depolymerization and RhoA delocalization from the cell membrane of bFGF-stimulated endothelial cells, which are also reversed by GGPP treatment.

The antiproliferative effect of cerivastatin was related to an arrest of the cell cycle in G1/S phase. This inhibition of cell proliferation is associated with an increase in the amount of p21Waf1/Cip1, a cyclin-dependent kinase inhibitor predominantly shown). In contrast, mice that received matrigel containing only bFGF exhibited an abundant and continuous vascular network in the connective tissue around the matrigel plugs (data not shown). The vessel index, which was determined by using the Weidner method also showed a significant decrease in the bFGF- and cerivastatin-conditioned matrigel compared with the bFGF matrigel (15.66 ± 0.71 versus 26.83 ± 1.63, respectively; P<0.001). Without the induction of bFGF, vessel spots were very low, even with control, without any significant changes in the presence or absence of cerivastatin (6 ± 0.50 versus 8.43 ± 0.63 for cerivastatin-containing matrigel and mock matrigel, respectively).

Discussion

Statins have demonstrated their ability to lower hypercholesterolemia and to prevent the occurrence of arterial events (coronary and cerebral). However, recent evidence suggests that the beneficial effects of statins may extend beyond their effects on serum cholesterol levels.
involved in G1/S transition, which is also reversed by GGPP. In the present study, we have also demonstrated that the increase in p21Waf1/Cip1 is caused by an increased synthesis and not by a decrease in proteasome-induced degradation. Indeed, cerivastatin also induced an increase in the p21Waf1/Cip1 mRNA. Our results are in agreement with the finding of Allal et al., who showed that the geranylgeranylation of RhoA is required for suppressing p21Waf1/Cip1 transcription. However, despite the absence of an effect of cerivastatin on basal endothelial cell proliferation, cerivastatin also induced an increase in p21Waf1/Cip1 on unstimulated endothelial cells. Therefore, it was assumed that the inhibitory effect of p21Waf1/Cip1 is efficient only on proliferative cells because of the sustained expression of D-type cyclins. This is in agreement with the observation that cerivastatin induced a potent inhibitory effect only on highly proliferative breast cancer cells.

We also investigated the hypothesis that the increase in p21Waf1/Cip1 was not sufficient to explain the antiproliferative effect induced by cerivastatin. To address this point, we were prompted to analyze whether cerivastatin inhibits FAK (phospho-Tyr397) and Akt (phospho-Ser473) activations. FAK is targeted in focal adhesion complexes and is a downstream effector of RhoA. Moreover, the activation of FAK leads to its association with PI3K, which is required for Akt stimulation, which is strongly implicated in mediating diverse cellular biological functions of angiogenic growth factors, including cell survival, proliferation, and angiogenesis. This hypothesis is supported by our investigations showing that cerivastatin inhibits the bFGF-induced FAK and Akt phosphorylation, which is explained by the loss of focal adhesion sites and RhoA inhibition.

Whatever the mechanism of action, the antiangiogenic activity of cerivastatin was directly confirmed in 2 in vivo models (1) the formation of neovessels in bFGF-enriched matrigel introduced subcutaneously in the mice and (2) the chick CAM. These results also indicate that the effect of cerivastatin is observed in several types of microvascular endothelial cells and not only in microvascular cell lines. Moreover, the antiangiogenic effect of cerivastatin was also confirmed by using other endothelial cells from the microvascular of bone marrow origin (human bone marrow endothelial cell; results are not shown).

Our results are in contrast with the recently published data of Kureishi et al., who reported that statins promote angiogenesis, a phenomenon attributed to Akt activation. This discrepancy with our findings could be attributed to the differences in our experimental models, inasmuch as Kureishi et al used endothelial cells without angiogenic factors. Under these conditions, we were unable to show any effect of cerivastatin. In fact, our results demonstrate that cerivastatin inhibits only the stimulatory effect of angiogenic factors on endothelial cells. This discrepancy could also be due to the difference of the endothelial cell origin, inasmuch as we used microcapillary endothelial cells, but these authors used human umbilical vascular endothelial cells or bovine aortic endothelial cells, which are representative of the microvasculature. Moreover, the loss of adhesion emphasizes the involvement of Akt inactivation in cerivastatin-treated endothelial cells, as previously discussed. In addition, the present results are in agreement with the recent observation that Rho confines the expression of cyclin D1 to the mid-G1 phase of the cell cycle.

In conclusion, we have demonstrated that cerivastatin inhibits the in vitro microcapillary endothelial cell proliferation induced by angiogenic growth factors and in vivo angiogenesis. This inhibitory effect is dependent on RhoA inhibition. Multiple mechanisms have been reported that can connect the inhibition of RhoA with the antiangiogenic activity of cerivastatin, including the increase in p21Waf1/Cip1 and the inhibition of the mitogenic signaling pathways downstream from FAK and PI3K/Akt. Whatever the mechanisms involved in the antiangiogenic activity of cerivastatin, the present findings suggest that it could contribute to the protective effect induced by cerivastatin on atherothrombosis by inhibiting the development of atherosclerosis and complications due to plaque rupture. This is in agreement with the observation that statins tend to inhibit the destabilization of the plaque responsible for its rupture.

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References


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Auntreated

cerivastatin 10 ng/ml
cerivastatin 25 ng/ml

B

control OSM

G0/G1 phase cells (%)

control OSM

S phase cells (%)

control OSM

G2/M phase cells (%)

cerivastatin 10 ng/ml
cerivastatin 25 ng/ml
**Figure II:** Effects of cerivastatin on RhoA delocalization from cell membrane and on actin stress fibers depolymerization are reversed by GGPP.

The localization of RhoA (green) and the organization of the actin cytoskeleton (red) were analyzed by confocal microscopy on the bFGF-stimulated endothelial cells after a 24-hour incubation with 25 ng/ml cerivastatin. Addition of GGPP with cerivastatin resulted in nearly complete localization of RhoA at the cell membrane and in reorganization of actin stress fibers. Scale bar = 10 µm.
**Figure I:** C ervastatin induced a decrease in cell progression at G0/G1 phase of HMEC-1 cell cycle.

Cell cycle distribution was studied by flow cytometry according to Vindelov's technique. OSM-stimulated HMEC-1 were treated for 18 h (A-C) with cerivastatin (10 and 25 ng/ml). Results of 6 experiments in duplicate are expressed as the percentage of endothelial cells in each cell cycle phase ± SEM (* P<0.05, ** P<0.01, *** P<0.001 as compared with untreated cells, n=6).
Materials and methods

Cytokines and cerivastatin

R&D Systems (Minneapolis, MN) supplied recombinant human OSM, VEGF and bFGF. Cerivastatin was kindly provided by Bayer-Pharma (Puteaux, France).

Cell culture

The HMEC-1 (human microvascular endothelial cells-1) cell line was provided by Dr. Ades (Centers for Disease Control and Prevention, Atlanta, GA). HMEC-1 are representative of the microvasculature and have properties similar to those of the original primary culture. HMEC-1 were cultured in MCDB-131 medium (Sigma, France), supplemented with 15% fetal calf serum (FCS), 100 IU/ml penicillin, 100 µg/ml streptomycin, 10 ng/ml epidermal growth factor (Euromedex, France) and 1 mg/ml hydrocortisone (Pharmacia-Upjohn, France).

Cell proliferation assay

For the proliferation assay, we used the minimal concentration of FCS (7.5%) to allow sufficient viability of endothelial cells. Briefly, after trypsinization, the cells were seeded at a concentration of $5 \times 10^4$ HMEC-1s in each well of 24-well plates (Nunc, Denmark) and then incubated without or with cytokines (2.5 ng/ml OSM, 25 ng/ml bFGF or 20 ng/ml VEGF). Cerivastatin was added at concentrations indicated in the results section. After incubation for 3 days, cells were detached by trypsin (0.05%, wt/vol.; Sigma), resuspended in Isoton II solution (Coulter, France) and counted in a particle counter (CoulterZ1, Coultronics). To assess whether inhibition of isoprenoids intermediates of cholesterol biosynthesis is involved in the cerivastatin effect, experiments were performed in presence of MVA (100 µM), FPP (10 µM) or GGPP (10 µM) (Sigma).
Apoptosis analysis

bFGF-stimulated endothelial cells were treated or not by cerivastatin (10 and 25 ng/ml) for 24 hours and the number of apoptotic cells was determined according to the protocol of Ormerod et al. Endothelial cells were stained with 1 µg/ml Hoechst 33342 (Sigma) for 4 min prior to Propidium Iodide (PI; 5 µg/ml; Sigma) and then analyzed using a Coulter Epics V Flow Cytometer with UV laser excitation (100mW) at 357 and 337 nm. Blue fluorescence (Hoechst 33342-DNA, between 430 and 530 nm) and red fluorescence (PI-DNA, above 630 nm) were measured for each endothelial cell.

Adhesion assay

2.10^6 HMEC-1s were seeded in each well of 6-well plates (Nunc, Denmark) in endothelial cell medium containing 2% FCS (concentration of FCS which allows cell survival but not cell proliferation). After total endothelial cell adhesion, cells were incubated in presence or absence of cerivastatin and stimulated with or without bFGF for 18 hours. Then, endothelial cells were trypsinized, washed in phosphate-buffered saline (PBS), resuspended in endothelial cell medium and left to adhere in 6-well plates for 12 hours. The number of non adherent endothelial cells was then determined by the count of cells present in the each supernatant.

Determination of cell cycle analysis

Cell cycle determination was performed according to Vindelov's technique. Cells were treated by 2.5 ng/ml OSM in presence or absence of cerivastatin during 18 hours. The percentage of cells in each cell cycle phase was determined by flow cytometry (EPICS XL-
MCL, Coulter). Experiments were performed with and without MVA, FPP or GGPP as indicated in the cell proliferation assay.

**Western blot analysis**

Western blot cell extracts were prepared by lysing cells with a cell volume of lysis buffer (triton 1 %, 1mM phenylmethylsulfonyl fluoride, 5 µg/ml leupeptin, 10 µg/ml aprotinin). After sonication and centrifugation, the protein yield was quantified using the Bradford assay. The cell lysates were mixed with sample buffer containing 2-mercaptoethanol and sodium-dodecyl-sulfate (SDS) and heated for 5 minutes at 95°C. Samples containing 50 µg total protein were subjected to SDS-polyacrylamide gel electrophoresis and then transferred to polyvinylidene difluoride membranes (Amersham, UK). Binding of the primary antibody against monoclonal p21\(^{Waf1/Cip1}\) (1:500, Transduction Laboratories, UK), polyclonal Akt or polyclonal Akt phospho-Ser 473 (1:1000, New England Biolabs, UK), monoclonal FAK (1:1000, Transduction Laboratories) and polyclonal FAK phospho-Tyr 397 (1:500, Euromedex, France) was detected with the enhanced chemiluminescence (ECL) detection system (Amersham) using horseradish peroxidase-conjugated secondary antibody (1:5000, Dako).

**RNA isolation and reverse transcriptase-polymerase chain reaction assay (RT-PCR)**

Cell were incubated in a 6-well plate (Nunc) with confluence and then incubated for 12 hours with or without bFGF in presence or absence of cerivastatin (10 and 25 ng/ml). Cells were then detached with a non-enzymatic cell dissociation solution (Sigma) and washed twice in PBS. Total RNA extraction was performed using SV total RNA isolation system (Promega) according to manufacturer’s instructions. Oligonucleotide primers were as follows:

\[ p21^{Waf1/Cip1} \ (5^\prime\text{-AGGCAGAAGATGTAGAGCGG, 5^\prime\text{-CCGAAGTCAGTTCCTTGTGG})} \]
β-actin specific primers (5’-ATCTGGCACCACACCTTCTACAATGAGCTGCG, 5’-CGTCATACTCCTGCTTGCTGATCCACATCTGC). RT-PCR was performed using the Access RT-PCR system (Promega, Madison, WI). For each reaction, (volume of 50 µl), the mixture containing 0.4 pg of total RNA, 50 pmol of each primer, 0.2 µM of dNTPs, 1 mM of MgSO4, 5 µl of 10X AMV/Tfl reaction buffer was prepared and denatured beforehand for 2 min at 94°C. When the temperature was reduced to 48°C, 2 U of AMV reverse transcriptase and 2 U of Tfl DNA polymerase were added in the reaction mixtures. An incubation for reverse transcription was performed at 48°C for 45 min. The PCR consisted of 40 or 29 cycles (respectively for p21Waf1/Cip1 and β-actin) of 1 min at 94°C, 30 s at 60°C and 1 min at 68°C. Finally, a step of extension at 68°C for 7 min improved the quality of the final product resulting in PCR amplification products of 638 bp for p21Waf1/Cip1 and 838 bp for β-actin which were then analyzed in agarose gel (1.5 %) electrophoresis.

**ELISA assay of p21Waf1/Cip1**

After a 24-hour incubation of both OSM-stimulated and unstimulated HMEC-1, cells were treated with or without FPP (10 µM) or GGPP (10 µM) in the presence or absence of cerivastatin (25 ng/ml). Cells were then lysed and the measurement of p21Waf1/Cip1 antigen in cell lysate was performed using an ELISA assay, according to the manufacturer’s instructions (Calbiochem, France).

**Confocal microscopy analysis of RhoA and actin filaments**

The confocal microscopy analysis of RhoA and actin filaments was performed on the bFGF-stimulated-HMEC-1 cells after a 24 hour-incubation with cerivastatin as previously described.5 RhoA was detected using first a monoclonal antibody against RhoA (Santa Cruz Biotechnology, CA) and second a fluoresceine isothiocyanate-conjugated anti-mouse IgG
(Immunotech, France). Actin filaments were visualized by tetra methyl rhodamine isothiocyanate-labeled phalloidin.

To assess whether inhibition of GGPP synthesis is involved in the cerivastatin effect, experiments were performed in presence or absence of GGPP (10 µM).

**In vivo angiogenesis assays**

1- matrigel model

To analyze the anti-angiogenic properties of cerivastatin, a matrigel model described by Passaniti *et al.* was used.⁶ 300 µL of matrigel (11.7 mg/ml; Becton Dickinson, France) in liquid form maintained at 4°C was mixed with or without 1 µg of bFGF in the presence or absence of cerivastatin (100 µg) and then injected subcutaneously in the dorsa of six-week-old Swiss *nu/nu* female mice (Charles Rivers, France) using a 24-gauge needle. All treatment groups contained 5 mice. Ten days after injection, tissue containing the matrigel plugs including the adjacent skin were removed, fixed overnight in absolute ethyl alcohol and embedded in paraffin. Matrigel sections of 5-µm thickness were prepared. The endogenous peroxidase activity was quenched by 3% H₂O₂ for 10 minutes. Neovessels were visualized by incubation of matrigel sections with a rat antibody against mouse PECAM-1 (platelet-endothelial cell adhesion molecule 1; Pharmingen, France) and then with a biotinylated goat anti-rat IgG antibody. After washing, sections were incubated with streptavidin-peroxydase and the vessels were revealed by peroxydase substrate diaminobenzidin. Meyer's hematoxylin was used for counterstaining. The vascularization level around the matrigel plugs was evaluated as described previously by Weidner *et al.*⁷
2- Chick CAM model

Fertilized chick embryos (Ferme Avicole François Haas, Kaltenhouse, France) were preincubated at 38°C with 80% humidity for 3 days in ovo. The shells were then cracked open aseptically and the embryonated eggs were placed in plastic culture dishes (Merck-Eurolab, France) according to an established shell-less culture technique exposing the CAM to direct access for experimental manipulation. At days 6 of embryonic development, angiogenic areas were cercled with a silicon ring (Weber Métaux, France) and PBS or cerivastatin (50 ng) in a final volume of 10 µl were placed inside the rings. The embryos were then placed into the incubator to induce spontaneous angiogenesis and were treated daily. After 10 days of embryonic development, treated areas were photographed.

Statistical analysis

Both in vitro and in vivo results were statistically analyzed using a two-tailed non-parametric Mann-Whitney test using the InStat software (Sigma). The results are expressed as mean value ± standard error of the mean (SEM). P<0.05 was considered significant.

References


