HMG-CoA Reductase Inhibitors Reduce Senescence and Increase Proliferation of Endothelial Progenitor Cells via Regulation of Cell Cycle Regulatory Genes

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Abstract—Endothelial progenitor cells (EPCs) play an important role in postnatal neovascularization of ischemic tissue. Ex vivo expansion of EPCs might be useful for potential clinical cell therapy of myocardial ischemia. However, cultivation of primary cells leads to cellular aging (senescence), thereby severely limiting the proliferative capacity. Therefore, we investigated whether statins might be able to prevent senescence of EPCs. EPCs were isolated from peripheral blood and characterized. After ex vivo cultivation, EPCs became senescent as determined by acidic β-galactosidase staining. Atorvastatin or mevastatin dose-dependently inhibited the onset of EPC senescence in culture. Moreover, atorvastatin increased proliferation of EPCs as assessed by BrdU incorporation and colony-forming capacity. Whereas geranylgeranylpyrophosphate or farnesylpyrophosphate reduced the senescence inhibitory effect of atorvastatin, NO synthase inhibition, antioxidants, or Rho kinase inhibitors had no effect. To get further insights into the underlying downstream effects of statins, we measured telomerase activity and determined the expression of various cell cycle regulatory genes by using a microarray assay. Whereas telomerase activity did not change, atorvastatin modulated expression of cell cycle genes including upregulation of cyclins and downregulation of the cell cycle inhibitor p27Kip1. Taken together, statins inhibited senescence of EPCs independent of NO, reactive oxygen species, and Rho kinase, but dependent on geranylgeranylpyrophosphate. Atorvastatin-mediated prevention of EPC senescence appears to be mediated by the regulation of various cell cycle proteins. The inhibition of EPC senescence and induction of EPC proliferation by statins in vitro may importantly improve the functional activity of EPCs for potential cell therapy. (Circ Res. 2003;92:1049-1055.)

Key Words: aging ■ telomerase ■ statins ■ progenitor cells ■ angiogenesis

Increasing evidence suggests that endothelial progenitor cells (EPCs) play a crucial role in neovascularization of ischemic tissue.1-2 EPCs are considered to derive from hematopoietic stem cells, which are positive for CD34.3,4 Ischemia can trigger the release of bone marrow–derived CD34-positive cells into the periphery.5 In animal experiments, CD34-positive cells were shown to home to sites of ischemia and express endothelial markers like KDR.6 Moreover, injection of CD34-positive cells or cultivated EPCs enhances neovascularization, associated with an improvement of cardiac function.1 Thus, one may consider the use of EPCs for potential cell therapy to augment vascularization in patients with ischemic heart disease.7 However, due to the limited number of EPCs in the circulating blood (<0.05% of leukocytes),8,9 ex vivo expansion of EPCs appears to be necessary. Proliferation of primary human cells is limited by the capacity to divide and the onset of senescence.10 Cellular aging or senescence is characterized by cell cycle arrest and can be triggered by different pathways.11 Replicative senescence results from a count down of the intrinsic mitotic clock. The mitotic counter mechanism is reflected by telomere shortening.12 With each cell division, telomeres become shorter. This telomere shortening contributes to the finite number of cell divisions of somatic cells. To maintain a certain telomere length, the enzyme telomerase, a ribonucleoprotein with reverse transcriptase activity, adds telomeric repeats at the linear end of eukaryotic chromosomes.13 Premature senescence, in contrast, describes the induction of senescence by extrinsic factors such as oncogenic ras, DNA damage, oxidative stress or cumulative stress induced by in vitro culture.11,13 Furthermore, a senescent phenotype can also be induced by expression of cyclin-dependent kinase inhibitors (CDKIs).11 The senescence phenotype is indistinguishable, irrespective of the inducer. It is characterized by a high
frequency of nuclear abnormalities, positive staining for β-galactosidase activity at pH 6.0.11,14 and can be associated with an increase in expression of cell cycle inhibitory proteins such as p27Kip1 or p21Cip/Waf1.15,16

In the present study, we investigated the regulation of senescence and proliferation in EPCs. Because we and others previously demonstrated that HMG-CoA reductase inhibitors increased the number of EPCs,8,17,18 we further elucidated a potential effect of statins on EPC senescence. Our data demonstrate that statins potently prevent the onset of EPC senescence and promote proliferation and colony formation ex vivo. The effects of statins appear to be largely independent on telomerase activity, but involve the transcriptional regulation of multiple cell cycle regulatory proteins.

Materials and Methods

Materials
Ly294002 (Biomol), wortmannin (Alexis), mevalonate (Fluka), and LNMA (Alexis) were preincubated 30 minutes before statin stimulation. VEGF, HA1077, and Y27632 were purchased from Calbiochem, and atorvastatin was kindly donated by Goedecke/Parke-Davis, Freiburg, Germany. Mevastatin was activated as previously described.19 Geranylgeranylation phosphatase and farnesylpyrophosphatase were from Sigma.

Cell Culture
Mononuclear cells were isolated by density gradient centrifugation with Bicoll from peripheral blood of healthy volunteers according to Vasa et al.9 Immediately after isolation, 4×10⁶ mononuclear cells were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; CellSystems, St Katharinen, Germany) supplemented with EGM SingleQuots and 20% FCS.

Colony Assay
After 4 days of culture, adherent cells were gently detached with EDTA. Cells (1×10⁵) were seeded in methylcellulose plates (Methocult GF H4434, CellSystems) with 100 ng/mL human recombinant VEGF. Plates were studied under phase contrast microscopy, and colonies were counted after 10 days of incubation by two independent investigators.

Acidic β-Galactosidase Staining
After 4 days of culture, adherent cells were detached and seeded in methylcellulose plates as described above. After 7 days, cells were fixed for 10 minutes in 2% formaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline (PBS), and incubated for 12 hours at 37°C without CO₂, with fresh β-Gal staining solution: 1 mg/mL of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium ferricyanide, and 2 mmol/L MgCl₂; pH 6.0. Cells were counterstained with 4’,6’-diamidino-phenylindole (DAPI; 0.2 μg/mL in 10 mmol/L Tris-HCl, pH 7.0, 10 mmol/L EDTA, 100 mmol/L NaCl) for 10 minutes to count the total cell number. The absolute numbers of β-galactosidase—positive cells were counted out of 1000 cells.

Telomerase Assay
EPCs were washed in PBS, and the pellet was lysed with 30 μl lysis buffer for 30 minutes at 4°C as previously described.20 Proteins were centrifuged for 20 minutes at 10000g, and protein concentrations were determined in the supernatant using the Bradford assay. Telomerase activity was measured with 2 μg protein by the TeloTAGGG Telomerase PCR ELISA kit according to the manufacturers instructions (Roche Molecular Biochemicals).

FACS Analysis
Adherent cells were gently scraped off using cell scrapers, washed in PBS, and incubated in PBS/1% BSA/1% mouse serum in the presence of the following antibodies. Staining of mouse anti-human VE-cadherin, (Santa Cruz Biotechnology), anti-human vWF antibody (BD Pharmingen), and KDR (Dianova) was visualized using RPE-conjugated goat anti-mouse F(ab’)2 (DAKO), ICAM-1 (BD Pharmingen), α, (CD51) (Dianova), and E-selectin (CD62E) (Dianova) were used directly FITC-conjugated.

Cell Cycle Analysis
Adherent cells were incubated with BrdU (10 μmol/L) for 48 hours. Adherent cells were detached with trypsin, washed in PBS, and incubated with 20 μL anti-BrdU-FITC for 20 minutes and with 2.5 μL 7AAD for 15 minutes according to the manufacturer (Pharmingen, BrdU Flow Kit). Analysis was performed using a FACS SCAN flow cytometer and Cell Quest software (BD Biosciences).

Microarray Analysis
Gene expression profiling was performed with the gene chip expression assay. The protocol for sample preparation and microarray processing is available from Affymetrix. Data were analyzed with the software GeneSpring version 3.0 (Silicon Genetics) as previously described.21

Western Blot Analysis
EPCs were incubated with 60 μL lysis buffer as previously described.15 Proteins (50 μg/lane) were loaded onto SDS-polyacrylamide gels and blotted onto PVDF membranes. Western blots were performed using antibodies directed against cyclin F, cyclin D, E2F1, cdk4 (all Santa Cruz) or cyclin A, PCNA, p21Cip/Waf, and p27Kip1 (all BD Biosciences). Enhanced chemiluminescence was performed according to the instructions of the manufacturer (Amer sham, Germany). Blots were reprobed with tubulin (Neomarkers).

Statistical Analysis
Data are expressed as mean±SEM from at least 3 independent experiments. Statistical analysis was performed by the two-tailed t test or ANOVA for multiple comparisons.

Results

HMG-CoA Reductase Inhibitors Prevent EPC Senescence and Increase Proliferation of EPCs
EPCs were generated from peripheral blood mononuclear cells as previously described.9,17 To assess the onset of senescence, acidic β-galactosidase was detected as a biochemical marker for acidification typical for the onset of cellular senescence.11,14 Cultivation of EPCs resulted in an increase in acidic β-galactosidase—positive cells after prolonged cultivation (Figure 1A). Coincubation with atorvastatin significantly inhibited the increase in β-galactosidase—positive cells (Figures 1A and 1B). The inhibition of EPC senescence occurred dose-dependent with a maximal inhibitory effect achieved at 0.1 μmol/L (Figure 1C). Mevastatin also inhibited the onset of EPC senescence to a similar extent (Figure 1D), suggesting a class effect of statins. To characterize the phenotype of the adherent cells, we analyzed the expression of endothelial marker proteins. EPCs cultivated in the presence or absence of atorvastatin revealed a similar expression pattern of the endothelial marker proteins (Figure 1E).

Consistent with previous findings,17,18 the number of EPCs also increased after atorvastatin incubation (data not shown). However, the absolute number of senescent EPCs was also significantly decreased to 16.2±2.3% β-galactosidase—posi-
tive EPCs/high power field after atorvastatin treatment. These data demonstrate that the decrease in the percentage of senescent cells was not caused by a relative increase in cell number.

Having demonstrated that atorvastatin delayed the onset of senescence, we examined whether that translates into an increase in proliferation and clonal expansion. As shown by FACS analysis of BrdU-labeled cells, atorvastatin induced a 2-fold increase in the number of cells in the S-phase (Figure 2A). To investigate the clonal expansion potential of the cultivated EPCs, we further performed an outgrowth assay. For this purpose, EPCs were cultivated for 4 days in the presence or absence of atorvastatin. Then, cells were detached, and 1×10⁵ EPCs were seeded in methylcellulose plates. As shown in Figures 2B and 2C, the number of colonies was significantly higher in EPCs that had been pretreated with atorvastatin. The endothelial phenotype of the outgrowing cells was confirmed by showing DiLDL uptake and lectin staining (Figure 2D).

**Intracellular Signaling of HMG-CoA Reductase Inhibitors**

Statins inhibit the HMG-CoA reductase and, thereby, prevent formation of mevalonate and the downstream products farnesylpyrophosphate (FPP) and geranylgeranylpyprophosphate (GGPP), which finally results in the inactivation of the Rho kinase. To investigate the involvement of these intermediates, we coincubated EPCs with mevalonate, GGPP, or FPP. As shown in Figure 3A, mevalonate completely reversed the inhibitory effect of atorvastatin on EPC senescence. Likewise, GGPP and FPP significantly prevented atorvastatin-induced inhibition of EPC senescence (Figure 3A). In contrast, senescence of EPCs was not prevented by two pharmacological inhibitors of the Rho kinase (Figure 3A), indicating that statins influence EPC senescence via isoprenylation, but independent of the Rho kinase.

Recent studies demonstrate that statins stimulate the PI3K/Akt pathway, which is known to regulate senescence of mature endothelial cells. Therefore, we investigated the
effect of the PI3K inhibitor Ly294002. After incubation with Ly294002 for 4 days, no attached cells were detectable in the presence or absence of statins, concomitant with an increase in apoptosis (data not shown). Thus, the PI3K pathway represents a potent survival signal in EPCs. A specific role of the PI3K pathway for EPC senescence cannot be assessed in the long term cultivation assay required to study cellular aging.

Statins are known to posttranscriptionally activate the endothelial NO synthase via the PI3K/Akt pathway and additionally reduce oxidative stress via regulation of isoprenylation. Because oxidative stress is well-established to contribute to cellular senescence and NO was recently demonstrated to prevent senescence of mature endothelial cells, we investigated the contribution of nitric oxide and reactive oxygen species for the regulation of EPC senescence. However, neither the antioxidant N-acetyl-cysteine (NAC) nor the NO donor SNAP did prevent the onset of EPC senescence (Figure 3B). Likewise, inhibition of the NO synthase by N’-monomethyl-L-arginine (LNMA) did not reverse the inhibitory effect of atorvastatin (Figure 3B), suggesting that the regulation of EPC senescence is independent of NO and reactive oxygen species.

Regulation of Telomerase and Cell Cycle–Related Genes
Cellular senescence is critically influenced by the telomerase, which elongates telomeres, thereby counteracting telomere length reduction induced by each cell division. Therefore, we measured telomerase activity in EPCs. EPCs revealed a basal telomerase activity, albeit at a low level (Figure 4A). Incubation of EPCs with atorvastatin did not affect telomerase activity (Figure 4A). Furthermore, the localization of the catalytic subunit of the telomerase was not altered by atorvastatin incubation (data not shown). These data indicate that statins act independent of telomerase.
To get further insights into the regulation of cell cycle regulatory genes, we performed a microarray, which allows for the detection of about 12 000 genes. Microarray analysis was performed after 10 hours of atorvastatin treatment, thus avoiding potential confounding effects of increased proliferation, which was observed after >24 hours. Cluster analysis of the cell cycle regulatory genes revealed that the expression of more than 10% of the analyzed cell cycle genes including cyclins and PCNA are increased after statin incubation (Figure 5A). Moreover, the expression of the cell cycle inhibitory protein p27 was reduced (Figure 5A). The modulation of the expression of some cell cycle regulatory proteins by atorvastatin was further confirmed on the protein level (Figure 5B). Thereby, atorvastatin increased the expression of cyclin A, cyclin F, and PCNA to 2.1±0.5-, 1.9±0.1-, and 1.5±0.06-fold, respectively. Consistent with a downregulation of p27 mRNA levels, protein expression was dose-dependently reduced to 53.1±5.7% after atorvastatin incubation for 24 hours, whereas expression of another cyclin-dependent kinase inhibitor, p21, was not affected (Figures 5B and 5C). Incubation with Ly294002 or wortmannin but not with Rho kinase inhibitors for 24 hours largely prevented the effect of atorvastatin on cyclin A, cyclin F, and p27 expression (Figures 5B through 5F and data not shown), suggesting that the PI3K pathway plays a major role for the modulation of expression of cell cycle regulatory genes. Controls confirmed that the short-term incubation with Ly294002 for 24 hours did not affect cell viability (apoptosis: 126±11% compared with untreated controls).

**Discussion**

The results of this study demonstrate that ex vivo cultivation of EPCs leads to rapid onset of EPC senescence. EPC senescence was associated with a very low proliferative capacity and profoundly impaired clonal expansion potential. Consistent with these findings, the activity of telomerase was very low in EPCs. At a first glance, these findings might be surprising, because stem or progenitor cells are viewed as highly potent regenerating cells with a high proliferation potential. However, one should keep in mind, that after mobilization of stem and progenitor cells into the circulation, these cells should preferentially home to sites, where they are needed, and should selectively contribute to cellular regeneration at these sites. The differentiation and proliferation state, therefore, may essentially be dependent on the cellular environment, which includes cell-to-cell communication and growth factor support. This may explain that senescence is rapidly increasing under ex vivo culture conditions, which lack cell-to-cell contact and potential autocrine factors required for functional differentiation of progenitor cells. Previous studies demonstrated that purified circulating hematopoietic CD34-positive progenitor cells exhibit a lower proliferation rate and reduced expression of various cell cycle stimulatory genes in comparison with CD34-positive cells, which were isolated directly from the bone marrow.27 The functional importance of the cellular environment is further supported by demonstrating that co-culturing peripheral blood CD34-positive cells with human endothelial cells transfected with various hematopoietic growth factors significantly enhanced the ex vivo expansion of progenitor cells.28 These data suggest that the cumulative trauma of in vitro culture (“culture shock”) and an impaired environment trigger premature senescence of EPCs.

Our data provide first evidence that ex vivo incubation with statins not only increased the number of EPCs,17,18 but also delayed the onset of cellular senescence. The mechanisms, by which statins are preventing the onset of cellular senescence and increasing the proliferative capacity of EPCs, appear to involve the geranylgeranyl pathway, because mevalonate, GGPP, and FPP reversed the senescence inhibitory effect of statins. Inhibition of geranylgeranylation was shown to inactivate the Rho kinase.19 However, pharmacological inhibitors of the Rho kinase did not affect EPC senescence, excluding that inhibition of the Rho kinase mediates the senescence inhibitory effect of statins. Likewise, statins act independent of NO and reactive oxygen species, although NO and ROS have been shown to play a key role in regulation of senescence of mature endothelial cells.20,26

However, our data demonstrate that statins transcriptionally modulate the expression of multiple cell cycle regulatory proteins. Several cell cycle-promoting proteins are upregulated on mRNA and protein levels. These proteins included cyclins A, D, and F as well as the cofactor for the DNA polymerase PCNA. One may speculate that the increased expression is secondary to a pro-proliferative effect of statins. However, this appears unlikely, because the gene array analysis was performed after 10 hours at a time point where no increase in S-phase was detectable. Moreover, the expression of the cell cycle inhibitory protein p27 was reduced after statin treatment. Our data further suggest that the PI3K/Akt pathway essentially contributes to the statin-induced modulation of gene expression. The PI3K/Akt pathway is activated by statins22 and is known to transcriptionally and posttranscriptionally modulate cell cycle progression.29,30 The transcriptional regulation of p27 by Akt has been attributed to the functional inactivation of the FOXO subfamily of forkhead transcription factors.31 Indeed, the forkhead transcription factor AFX (FOXO4) is expressed in EPCs and is phosphorylated and, thereby, inactivated by statins (C.U., PhD, oral communication, 2002). These data may suggest that the statin-mediated downregulation of p27 is controlled by Akt-
dependent inactivation of forkhead transcription factors. Importantly, downregulation of p27 has been shown to be involved in rescue of fibroblast senescence induced by PI3K inhibitors, providing also an explanation for the inhibition of senescence-like growth arrest in statin-treated EPCs. The signaling pathways, by which statins increase the mRNA level of cyclins and PCNA, is less clear. Although Akt has been shown to induce posttranscriptional stabilization of cyclin D via inhibition of GSK-3, the transcriptional regulation of cyclin mRNA expression has not been demonstrated. Further studies are required to elucidate the molecular mechanisms underlying these effects.

Taken together, statins modulate expression of various cell cycle regulatory proteins in EPCs via the PI3K pathway. The increase of cell cycle promoting proteins, which are essential for cell cycle progression, concomitant with a reduction of the cell cycle inhibitory protein p27 may facilitate cell cycle progression and, thereby, prevent the onset of replicative senescence. Although the short-term treatment of EPCs with the PI3K inhibitor Ly294002 clearly prevented the changes in protein expression, we could not proof a causal involvement of the PI3K for the senescence inhibitory effect of statins, because long term incubation of Ly294002 also induced apoptosis of EPCs. This is consistent with the finding that
activation of Akt by statins prevents apoptosis of EPCs.\textsuperscript{18}
Thus, activation of the PI3K/Akt pathway by statins may have multiple protective effects on EPCs, including the increase in number, the inhibition of apoptosis, the improvement of functional activity, and the prevention of senescence.

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