Statins Enhance Migratory Capacity by Upregulation of the Telomere Repeat-Binding Factor TRF2 in Endothelial Progenitor Cells

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Background—Cultivation of endothelial progenitor cells (EPCs) leads to premature replicative senescence, limiting ex vivo expansion for potential clinical cell therapy. Recent studies have linked senescence to the dysfunction of telomeres, the “ends” of chromosomes, via the so-called mitotic clock or culture-induced stress. The purpose of this study was to elucidate a possible role of telomere biology in the functional augmentation of EPCs by statins.

Methods and Results—Human EPCs were isolated from peripheral blood. Using flow cytometry after fluorescence in situ hybridization with a telomere-specific (C,T,A,T)3 peptide nucleic acid probe (Flow-FISH), we found mean telomere length in untreated EPCs from healthy subjects to range between 8.5±0.2 and 11.1±0.5 kb with no change over 6 days of culture, excluding telomere erosion as one cause for premature senescence. Although mean telomere length did not differ between statin-treated and untreated EPCs, atorvastatin (0.1 μmol/L) and mevastatin (1.0 μmol/L) both led to a more than 3-fold increase in the expression of the telomere capping protein TRF2 (telomere repeat-binding factor), as shown by immunoblotting, whereas quantitative reverse transcription–polymerase chain reaction demonstrated no increase in TRF2 mRNA. Telomere dysfunction of EPCs was also paralleled by a 4-fold increase in the DNA damage checkpoint-kinase 2 (Chk2). Conversely, statin cotreatment or overexpression of TRF2 completely suppressed Chk2 induction. Finally, overexpression of a dominant negative mutant of the TRF2 protein abrogated statin-induced enhancement of migratory activity down to baseline values.

Conclusions—Ex vivo culturing of EPCs leads to “uncapping” of telomeres, indicated by the loss of TRF2. Statin cotreatment of EPCs prevents impairment of their functional capacity by a TRF2-dependent, posttranscriptional mechanism. This is the first time a beneficial effect of statins on telomere biology has been described. (Circulation. 2004;110:3136-3142.)

Key Words: telomere ■ aging ■ stem cells ■ telomere repeat-binding factor 2
Here, we demonstrate that statins delay premature senescence in cultivated EPCs independently of mean telomere length. Statin-enhanced migratory activity of EPCs also depends in part on the induction of the telomere-capping protein TRF2. This is the first time a protective effect of statins on telomere biology has been described.

Methods

Materials
Human recombinant vascular endothelial growth factor (VEGF), macrophage-colony stimulating factor, granulocyte-macrophage colony-stimulating factor, and interleukin-4 were purchased from Cell Concepts. Antennapedia internalization sequence peptide was synthesized by BioSynth.

Cell Culture
Mononuclear cells were isolated by density-gradient centrifugation with Ficoll from peripheral blood of healthy human volunteers as described previously.28 Immediately after isolation, total mononuclear cells (8×10⁶ cells/mL medium; cell density, 2.5×10⁶ cells/cm²) were plated on culture dishes coated with human fibronectin (Sigma) and maintained in endothelial basal medium (EBM; Clonetics) supplemented with EGM SingleQuots, VEGF (100 ng/mL), and 20% FCS. More than 90% of adherent EPCs show endothelial characteristics, as demonstrated by Dil-acetylated LDL uptake and lectin binding and immunostaining of typical endothelial marker proteins, including VEGF receptor (VEGFR2) (KDR) (ReliaTech), endoglin (CD105) (NeoMarkers), and von Willebrand factor (Oncogene). Using FACS analysis, we found 94% of EPCs to stain positive for KDR, 76% for CD105, and 72% for von Willebrand factor.

Q-FISH
Endothelial cells or EPCs were incubated for 24 hours with 100 ng/mL colcemide. Nuclei were isolated by hypotonic swelling in a 60-mmol/L KCl solution and fixed in a 2:1 solution of methanol/glacial acid. Hybridization was performed with a Cy3-conjugated FITC-labeled fluorescent beads (DAPI; 2 μg/mL).

Determination of Human Telomere Length by Flow-FISH
The average length of telomere repeats at chromosome ends in individual peripheral blood leukocytes was measured by Flow-FISH as previously reported. EPCs were detached by trypsin, and the cell suspension was centrifuged for 5 minutes at 800 g. The supernatant was removed, and the cell pellets were washed twice with PBS. A total of 2×10⁶ EPCs and 1×10⁵ calf thymocytes (as an internal calibration standard) were resuspended together in the following hybridization mixture: 20 mmol/L Tris, 20 mmol/L NaCl, 1% BSA, 75% formamide (Invitrogen), and 0.3 μg/mL telomere-specific FITC-conjugated (C,TA₃)ₙ peptide nucleic acid (PNA) probe (Dako) according to the manufacturer’s instructions. Counterstaining was performed with 4',6-diamino-2-phenylindole (DAPI; 2 μg/mL).

Whole-Cell Extracts
Cells were washed 3 times in cold PBS and then lysed for 30 minutes at 4°C in lysis buffer containing 20 mmol/L Tris-HCl (pH 7.4), 1 mmol/L EDTA (pH 8.0), 1 mmol/L EGTA, 150 mmol/L NaCl, 1% Triton, 2.5 mmol/L Na-pyrophosphate, 1 mmol/L β-glycerophosphate, 1 mmol/L Na-orthovanadate, and the following protease inhibitors: 200 mmol/L PMSF and 1 μg/mL leupeptin. After centrifugation at high speed, the supernatant was collected and the protein content of all samples was determined using the Bio-Rad protein assay with γ-globulin as a standard.

Western Blot Analysis
Electrophoresis was performed on 12% SDS-polyacrylamide gels loading 30 μg of protein per lane. After transfer to a 0.2-μm polyvinylidene difluoride membrane (Bio-Rad), the membranes were blocked in 3% BSA (in PBS, pH 7.5, and 0.2% Tween 20) for 1 hour and incubated overnight with the indicated primary antibody (Ab) at 4°C. Each Ab incubation period was followed by 1 hour of membrane washing in 0.1% Tween-20 in PBS. Detection was performed by use of a horseradish peroxidase–linked anti-rabbit (1:7500 in PBS/1% Tween 20/2% nonfat milk, anti-goat (1:5000), or anti-mouse (1:5000 in PBS/1% Tween 20/2% nonfat milk) (secondary) Ab (Amersham) and the Amersham enhanced chemiluminescence system (ECL kit). The following primary Abs were used: β-actin mouse monoclonal Ab (Sigma A5441; 1:8000), TRF2 H-300 rabbit polyclonal Ab (Santa Cruz sc-9143; 1:250), TRF2 goat polyclonal Ab (Santa Cruz sc-8528; 1:250), Checkpoint-kinase 2 (Chk2) mouse monoclonal Ab (Santa Cruz sc-17747; 1:250), green fluorescent protein (GFP) rabbit polyclonal Ab (Invitrogen 46-0092; 1:3000), and c-myc 3B11 mouse monoclonal Ab (Cell Signaling 2276; 1:500).

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction
Total RNA from 10⁶ EPCs was isolated by the guanidinium method using Qiagen’s RNA extraction kit. Quantification of TRF2 mRNA was performed by amplification of EPC cDNA in a 1-step reverse transcription–polymerase chain reaction (RT-PCR) using the LightCycler (Roche Diagnostics) real-time thermocycler according to the instructions of the manufacturer (Roche Diagnostics). The RT-PCR reaction was composed of 3.25 mmol/L Mn(OAc)₂, 1× LightCycler–RNA Master SYBR Green I, 100 ng mRNA, and 0.5 μmol/L of each primer in a final volume of 20 μL. The following oligonucleotide primers were used for amplification of human TRF2 cDNA: sense (5′→3′) CCCAAGAACAAGCGCATGAC and antisense (5′→3′) TTTCCTGCACCTCGCCATTGAC. Amplification was performed with 45 cycles and an annealing temperature of 61°C. Copy numbers were calculated by the instrument software (Roche Diagnostics) from standard curves of an in vitro–transcribed cytokine mRNA. The specificity of the amplification reaction was determined by a melting curve analysis. PCR products were analyzed on a 2% agarose gel to confirm a 341-bp product.

Adenoviral Constructs and Infection of EPCs
Plasmids for human TRF2 and its dominant-negative truncation (TRF2Δ304) were provided by T. de Lange (Rockefeller University, New York, NY). Adenoviruses coexpressing enhanced GFP were generated by use of pAdTrack-cytomegalovirus and...
pShuttle-cytomegalovirus (provided by B. Vogelstein, Johns Hopkins Oncology Center, Baltimore, Md34,35) and have been described previously.24 If not otherwise stated, Antennapedia peptide (Antennapedia internalization sequence RQIAIWFQNR-MKWAA36) at a concentration of 0.5 mmol/L, purified adenovirus, and 100/H9262/L of OptiMem (Gibco) were incubated for 30 minutes at room temperature. The mixture was then added to freshly isolated mononuclear cells (see Cell Culture) in a 2.5-mL volume and left together with the cells until day 3. Adenoviral infection was validated by visualization of enhanced GFP under fluorescence microscopy.

Assessment of Migratory Capacity of EPCs
After 3 days of culture, progenitor cells were resuspended in EBM without FCS, containing 1% BSA. Then 5×10⁴ EPCs were placed in the upper chamber of a modified Boyden chamber (Falcon HTS Fluoro Blok insert, No. 351152, 8-μm pore size). The chamber was placed in a 24-well culture dish (Falcon, No. 353504) containing endothelial basal medium, 50 ng/mL VEGF, and 100 ng/mL stromal cell–derived factor (SDF)-1 (where indicated) for measuring the migratory capacity of EPCs. After 24 hours of incubation at 37°C, the lower side of the filter was washed with PBS and fixed with 2% paraformaldehyde. For quantification, cells were stained with Dil-acetylated LDL and were counted manually in 3 random microscopic fields per well.

Results
Lack of Telomere Erosion in Senescent EPCs
To address the possibility of telomere erosion as a cause of premature senescence and dysfunction in cultured EPCs, we performed the flow cytometric determination of the mean telomere length after fluorescence in situ hybridization (Flow-FISH) with labeled telomere probes as described previously.29–32,37 In situ telomere hybridization (Q-FISH) was performed to prove the specificity of the used (C3TA2)₃ PNA probe in EPCs (data not shown). EPCs were analyzed in interphase. Using Flow-FISH, we found the mean telomere length in EPCs generated from different blood donors to range between 8 and 11 kb, independent of statin cotreatment (Figure 1C). We also performed longitudinal studies on isolated mononuclear cells. Here, mean telomere length did not differ between freshly isolated mononuclear cells and EPCs on days 4 and 6 (Figure 1D). These results indicating no change in mean telomere length during cell culture argue against telomere erosion as a possible cause for premature senescence in cultured EPCs from healthy subjects. In addition, because mean telomere
length by itself can conceal small populations of critically shortened telomeres, we also specifically compared the potential effects of propagation and statins on the shortest length of telomeres in the Flow-FISH distribution and excluded a shift in the length distribution. Together, these findings would better support the concept of telomere dysfunction independent of critical telomere shortening, such as because of telomere un-capping.

**Statins Induce TRF2 by a Posttranscriptional Mechanism**

To investigate the possibility that statins might, instead, have salutary effects on telomere-associated proteins in cultured EPCs, we first examined the protein expression of the specific TRFs. Although expression of TRF1 did not change under statin treatment (data not shown), TRF2 was induced more than 3-fold by atorvastatin or mevastatin, in a dose-dependent manner, after 3 days of cultivation (Figure 2, A and B), and induction reached its maximum at 0.1 μmol/L and 1.0 μmol/L, respectively. Simvastatin (data not shown) had an identical effect on TRF2 expression at a concentration of ≥0.5 μmol/L. To obtain this effect, which was consistent among each of 3 healthy blood donors (Figure 2, A and B), statin incubation had to be initiated in the first 24 hours after isolation of mononuclear cells (Figure 2C). Addition of atorvastatin at later time points did not lead to an increase of TRF2 expression.

We previously showed that statins inhibit premature senescence in EPCs by preventing formation of mevalonate and the downstream products farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GPP). As shown in Figure 2D, mevalonate, FPP, and GPP partially repressed TRF2 induction by atorvastatin, indicating a mechanism involving isoprenylation. To test for induction of TRF2, we performed semiquantitative PCR using TRF2-specific oligonucleotide primers (Figure 2E). However, no statistical difference in TRF2 mRNA expression could be seen among the statin-treated and untreated cells, suggesting an enhancement of protein translation or stability rather than mRNA induction.

**Telomere Dysfunction of EPCs Leads to Chk2 Induction**

Because loss of the telomere-capping protein TRF2 leads to telomere dysfunction, as measured by activation of the DNA damage protein kinase pathway, we investigated this possible day 6 from EPCs. EPCs were treated either without (−) or with 0.1 μmol/L atorvastatin, added at indicated time points, where, only in lane marked with an asterisk (*), atorvastatin was already discontinued after 72 hours. D, Mononuclear cells were cultivated for 4 days in EBM with supplements and 20% FCS in presence or absence of atorvastatin (0.1 μmol/L), mevalonate (Mev; 200 μmol/L), farnesylpyrophosphate (FPP; 10 μmol/L), and geranylgeranylpyrophosphate (GPP; 10 μmol/L). E, Semiquantitative RT-PCR of TRF2 mRNA in EPCs using Lightcycler (Roche). Freshly isolated mononuclear cells were cultivated in EBM with supplements and 20% FCS in presence or absence of atorvastatin (AT; 0.1 μmol/L). DMSO (0.1%) served as a vehicle control. mRNA was isolated at 0 hours from mononuclear cells or after 24 and 48 hours from adherent cells. Data are mean±SEM, n=2. One of 3 representative experiments is shown.
downstream contender in TRF2 signaling. Activity of Chk2, a DNA damage kinase, increases accompanying partial loss of TRF2 in human myocardium during heart failure and is triggered in rat cardiomyocytes by interference with TRF2 function or expression.24 Indeed, Chk2 protein level in EPCs increases under culture conditions to more than 4-fold compared with levels in freshly isolated monocytes (Figure 3A), whereas atorvastatin, mevastatin, or simvastatin (data not shown) cotreatment completely abrogated Chk2 induction (23/100 vs 100% control; Figures 2A and 3B). Finally, overexpression of TRF2 wild-type protein in EPCs also prevented Chk2 upregulation (Figure 3C), suggesting that Chk2 is a downstream target of telomere dysfunction induced by lack of TRF2 in EPCs.

Enhancement of Migratory Activity by Statins Depends on TRF2 Expression

Finally, to provide evidence that the increase in TRF2 contributes to the increased functional capacity of EPCs elicited by statins, we expressed dominant-negative TRF2 in EPCs. To achieve this, freshly isolated mononuclear cells were infected with adenoviral constructs. The validity of viral vectors was confirmed by immunobLOTS in the day 3 EPCs (Figure 4A). Transduced EPCs were used on day 3 for migration assays, showing an increase in basal migratory activity after coculture with 0.1 μmol/L atorvastatin from 14.2±1.9 to 33.7±2.6 cells per high-power field (HPF; P<0.001; Figure 4B) and from 48±9.5 to 112±22 cells/HPF in response to SDF-1 as the chemoattractant (P<0.05; Figure 4C). Conversely, migratory activity in atorvastatin-treated
EPCs expressing dominant-negative TRF2 was reduced almost down to baseline (no statin pretreatment): 18.8±1.9 cells/HPF without SDF (P<0.001 versus Ad-GFP+atorvastatin; Figure 4B) and 53.7±3.9 cells/HPF with SDF attraction (P<0.01 versus Ad-GFP+atorvastatin; Figure 4C), respectively.

Discussion

The results of the present study demonstrate that statins induce expression of a telomere repeat-binding factor, TRF2, in cultured EPCs, thereby improving functional capacity and providing a potential explanation for the effect of statins on cell senescence.

Human telomeres are bound by 2 double-strand telomere binding proteins, TRF1 and TRF2, each of which recruits another set of interacting proteins. Removal of TRF2 triggers apoptosis or senescence and can result in fusion of the chromosome ends.33,38,39 Loss of TRF2, however, occurs in end-stage human heart failure and leads to telomere dysfunction.24 Overexpression of TRF2 and TERT is protective against oxidative stress in cultured cardiomyocytes.24 Similarly, we demonstrate that interference with TRF2 abolishes the statin-induced enhancement of the migratory capacity in EPCs. Of note, the migratory activity of EPCs reflects the most relevant parameter with regard to functional improvement after cell therapy for acute myocardial infarction.9 It is also possible that the induction of TRF2 by statins leads to “juvenation” of EPCs, because replicative senescence is delayed. Younger cells would migrate faster than older cells because of their age characteristics. In this case, TRF2 protein might correlate very well with cellular aging, thus exerting only an indirect role on cell migration.

Telomere length in human white blood cells shortens during aging.37 Interestingly, individuals with shorter white blood cell telomeres after adjustment for age show a >3-fold higher mortality rate from heart disease and more than 8-fold higher mortality rate from infectious diseases.40 Not only do environmental factors affect telomere length, but also, a higher mortality rate from heart disease and more than 8-fold increase in mean telomere length occurred as an explanation of how statins favorably affect senescence in human EPCs. Nevertheless, our data cannot exclude the possibility that telomeric shortening may occur at the level of individual telomeres at the chromosome ends.

Whereas ongoing progressive erosion of telomeres is the key factor in replicative senescence, more acute conditions also cause telomere dysfunction, including oxidative stress and other signaling cascades.13,43,44 An important consequence of telomere dysfunction is activation of the ATM-dependent DNA damage pathway. We found an important DNA damage checkpoint kinase, Chk2, to be upregulated in EPCs if cultured without atorvastatin. Chk2 can phosphorylate E2F1, p53, and the polo-like kinase 3 (Plk3, alternatively called Prk). Interestingly, Plk3 can be rapidly activated by reactive oxygen species in cultured fibroblasts.45–47 Chk2 itself is activated by phosphorylation, such as in cardiac myocytes failing because of telomere dysfunction.24 We found statins to suppress Chk2 induction in cultured EPCs if cells were treated within the first 24 hours. Statins have been proved to promote potent systemic antioxidant effects in vivo through suppression of different oxidation pathways, including the generation of myeloperoxidase-derived and nitric oxide–derived oxidants.48 This can be attributed primarily to the inhibition of isoprenylation, which was also a prerequisite for the induction of TRF2 by statins in our study. We have recently shown that statins can delay the onset of replicative senescence simply by inhibition of the nuclear export of TERT in endothelial cells.10

The recognition that premature replicative senescence of EPCs occurs in vitro does not necessarily imply that the same phenomenon will persist in vivo after their intravenous administration. Moreover, the remaining nonsenescent cells may be more than adequate to function in vivo.

In summary, loss of an essential telomere-capping protein and the impairment of functional capacity in EPCs during propagation in culture can be prevented by treatment with statins. Exogenous TRF2 reproduces the statin effect, and a dominant-interfering mutation blocks the statin effect, implicating TRF2 in this process, albeit through effectors that remain to be defined. This is the first time a beneficial effect of statins on telomere biology has been described.

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