Effect of intensive lipid-lowering therapy on telomere erosion in endothelial progenitor cells obtained from patients with coronary artery disease

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ABSTRACT
Telomere erosion of EPCs (endothelial progenitor cells) may be a key factor in endothelial cell senescence and is highly dependent on cellular oxidative damage. The aim of the present study was to investigate whether LLT (lipid-lowering therapy) with statins could attenuate EPC telomere erosion in patients with CAD (coronary artery disease). The study included 100 patients with stable CAD and 25 subjects without CAD as controls. CAD patients were randomized to 12 months of intensive LLT with atorvastatin or moderate LLT with pravastatin. EPCs were obtained from peripheral blood at baseline and after 12 months of statin therapy. Telomere length in EPCs was measured by FISH (fluorescence in situ hybridization) and oxidative DNA damage by flow cytometry of oxidized DNA bases. EPC telomere length was shorter in the CAD group than in the controls, and oxidative DNA damage to EPCs was higher in the CAD group compared with controls. After 12 months of therapy, changes in lipid profiles were greater in the intensive LLT group than in the moderate LLT group. Intensive LLT markedly increased EPC number and decreased oxidative DNA damage in EPCs (both \( P < 0.05 \)), with no change in telomere length. In contrast, moderate LLT did not change EPC counts or oxidative DNA damage, but showed telomere shortening (\( P < 0.05 \)). There was a weak negative correlation between changes in EPC number and LDL (low-density lipoprotein)-cholesterol levels after intensive LLT, whereas there was no correlation between them after moderate LLT. With in vitro culturing of EPCs subjected to oxidative stress, atorvastatin led to the prevention of EPC telomere shortening compared with pravastatin. In conclusion, the present study has demonstrated that intensive LLT may prevent EPC telomere erosion in patients with CAD, possibly contributing to the beneficial effects of intensive LLT in this disorder.

INTRODUCTION
The integrity and functional activity of the endothelial monolayer is essential for protection against the initiation of atherosclerosis [1]. Cardiovascular risk factors for atherosclerosis induce endothelial injury and lead to the formation of atherosclerotic lesions resulting in vascular inflammation [2]. Impaired endothelial function is a

Key words: atherosclerosis, coronary artery disease, endothelial progenitor cell, oxidative DNA damage, statin, telomere length.

Abbreviations: ACS, acute coronary syndromes; BSO, t-buthionine-(S,R)-sulfoximine; CAD, coronary artery disease; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; EBM-2, endothelial basal medium-2; EPC, endothelial progenitor cell; FISH, fluorescence in situ hybridization; HDL, high-density lipoprotein; LDL, low-density lipoprotein; acLDL, acetylated LDL; LLT, lipid-lowering therapy; mAb, monoclonal antibody; MFI, mean fluorescent intensity; PBMC, peripheral blood mononuclear cell; PerCP, peridinin–chlorophyll–protein complex; RTL, relative telomere length; t-BHP, t-butyl hydroperoxide; UEA-1, Ulex europeaus agglutinin-1.

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predictor for CAD (coronary artery disease) [3]. Therefore the maintenance of endothelial integrity is an important factor for preventing the development of CAD.

It has been reported that a decreased number of circulating EPCs (endothelial progenitor cells) is associated with the occurrence of cardiovascular events in patients at risk of CAD [4]. EPCs are regarded as having a key role in the maintenance of endothelial integrity and the replacement of apoptotic or damaged endothelial cells in response to various cardiovascular risk factors [5,6]. Cell division is associated with telomere shortening, leading to senescence once telomere length reaches a critical threshold [7]. Telomeres are composed of double-stranded repeats of the G-rich tandem DNA sequence TTAGGG extending over 6–15 kb at the end of eukaryotic chromosomes, and are necessary for both successful DNA replication and chromosomal integrity [8]. It has been demonstrated recently that telomeric DNA is not transcriptionally silent, but is being transcribed into telomeric repeat-containing DNA [9]. Our previous study has demonstrated telomere shortening and increased oxidative DNA damage of EPCs in patients with CAD compared with non-CAD patients [10]. Spyridopoulos et al. [11] have revealed a beneficial effect of statins on telomere biology in cultured EPCs. In randomized clinical trials, intensive LLT (lipid-lowering therapy) with atorvastatin had a beneficial effect by slowing the progression of coronary atherosclerosis compared with moderate LLT with pravastatin [12]. On the basis of these studies, this property of intensive LLT with statins may contribute to biologically relevant activities, including EPC telomere biology and angiogenesis. In the present study, our aim was to determine whether EPC telomere erosion could be modified by LLT with statins in patients with CAD.

MATERIALS AND METHODS

Study population

The present study included 100 consecutive patients with stable CAD who had no previous exposure to statin therapy. CAD was diagnosed on the basis of (i) the presence of a history of typical chest pain on effort, (ii) documented exercise-induced myocardial ischaemia, (iii) angiographically proven CAD, and (iv) the absence of ACS (acute coronary syndromes) for 3 months before blood sampling. Patients were excluded from the study if they had any clinical signs of acute infection, severe renal failure or rheumatoid disease, or if they were suspected of having a malignant or primary wasting disorder. Cell division is associated with telomere shortening, leading to senescence once telomere length reaches a critical threshold [7]. Telomeres are composed of double-stranded repeats of the G-rich tandem DNA sequence TTAGGG extending over 6–15 kb at the end of eukaryotic chromosomes, and are necessary for both successful DNA replication and chromosomal integrity [8]. It has been demonstrated recently that telomeric DNA is not transcriptionally silent, but is being transcribed into telomeric repeat-containing DNA [9]. Our previous study has demonstrated telomere shortening and increased oxidative DNA damage of EPCs in patients with CAD compared with non-CAD patients [10]. Spyridopoulos et al. [11] have revealed a beneficial effect of statins on telomere biology in cultured EPCs. In randomized clinical trials, intensive LLT (lipid-lowering therapy) with atorvastatin had a beneficial effect by slowing the progression of coronary atherosclerosis compared with moderate LLT with pravastatin [12]. On the basis of these studies, this property of intensive LLT with statins may contribute to biologically relevant activities, including EPC telomere biology and angiogenesis. In the present study, our aim was to determine whether EPC telomere erosion could be modified by LLT with statins in patients with CAD.

Quantification of circulating EPCs and cell culture enrichment of EPCs

PBMCs (peripheral blood mononuclear cells) were isolated from heparinized blood samples obtained from all subjects by Ficoll–Paque density gradient centrifugation. PBMCs were incubated with a PerCP (peridinin–chlorophyll–protein complex)-conjugated anti-(human CD34) mAb (monoclonal antibody) (Becton Dickinson) and a FITC-conjugated anti-[human KDR (kinase insert domain-containing receptor)] mAb (R&D Systems). The number of CD34- and KDR-double-positive cells among 1 × 10⁶ cells were counted using a FACScan analyser (Becton Dickinson).

PBMCs (8 × 10⁶) were plated on fibronectin-coated culture dishes (Sigma) and maintained in EBM-2 (endothelial basal medium-2) supplemented with EGM-2-MV-SingleQuots (Clonetics) containing 5 % (v/v) fetal bovine serum, 50 ng/ml human VEGF (vascular endothelial growth factor), 50 ng/ml human IGF-1 (insulin-like growth factor-1) and 50 ng/ml human EGF (epidermal growth factor). To exclude contamination with mature circulating endothelial cells, we carefully removed the culture supernatant 8 h after initial seeding and placed it into new fibronectin-coated culture dishes. Adherent cells were passaged once at day 4, and the endothelial phenotype was confirmed by cells positive for FITC-conjugated KDR (R&D Systems), PerCP-conjugated CD34 (Becton Dickinson) and PE-conjugated CD133 (Ancell Corporation) staining using a FACScan analyser (Becton Dickinson) [13]. After 8 days of culture, adherent cells of an endothelial lineage were identified by the concurrent binding of FITC-conjugated UEA-1 (Ulex europeaus agglutinin-1; Sigma) and the uptake of DiI (1,1′-dioctadecyl-3,3′,3′,3′-tetramethylindocarbocyanine perchlorate)-labelled acLDL [acetylated LDL (low-density lipoprotein); Invitrogen]. Two independent investigators evaluated the number of EPCs/mm² by counting DiI-labelled acLDL/UEA-1-double-positive cells in 15 randomly selected high-power fields using an inverted fluorescent microscope.

Determination of EPC telomere length

Telomere lengths of freshly isolated PBMCs and cultured EPCs were measured using a telomere peptide nucleic
acid kit/FITC for flow cytometry (Dako Cytomation). Telomere length of human lymphocytes measured by flow FISH (fluorescence in situ hybridization) correlated positively with results by Southern blot analysis ($R = 0.9$) [14]. RTL (relative telomere length) was determined by comparing isolated EPCs with bovine thymocytes as the internal control following the method described by Baerlocher et al. [15]. A total of $5 \times 10^5$ cells was resuspended and hybridized either with no probe or with a FITC-conjugated telomere peptide nucleic acid probe. Statistical data from these cells were then used to calculate the RTL of sample cells compared with control cells.

**Oxidative DNA damage**

An OxyDNA test kit (Biotrin) was used to evaluate oxidative DNA damage in EPCs, following the manufacturer’s recommendations. The probe is specific for 8-oxoguanine, which is formed during free radical damage to DNA and is a sensitive and specific indicator of oxidative DNA damage [16]. Briefly, $1 \times 10^6$ EPCs were incubated for 1 h at $37 \, ^\circ C$ with $50 \, \mu l$ of blocking buffer (Biotrin), and then incubated for 1 h at room temperature ($25 \, ^\circ C$) in the dark with $100 \, \mu l$ of FITC-labelled 8-oxoguanine probe. The cells were analysed by flow cytometry and the MFI (mean fluorescent intensity) of 8-hydroxyl 2′-deoxyguanosine was recorded.

**Cell culture with oxidant treatments**

Atorvastatin (Pfizer) and pravastatin (LKT Labs) were dissolved in DMSO (DMSO final concentration, 0.05%; Gibco). Atorvastatin and pravastatin were applied at 5-fold-diluted concentrations (atorvastatin, 0.02–0.5 $\mu M/l$; pravastatin, 2–50 $\mu M/l$). To induce oxidative stress, cultured EPCs from 20 patients with CAD before statin therapy (random sampling) were grown in EBM-2 lacking ascorbic acid and each statin or vehicle and exposed at each passage to 0.1 $\mu M/l$ BSO [L-buthionine-(S,R)-sulfoximine; Sigma] or PBS. After 8 days of culture, the percentage changes in telomere length in stimulated EPCs were compared with those in unstimulated EPCs.

**Statistical analysis**

All values are presented as means ± S.D. Kolmogorov–Smirnov analysis was performed to assess data distribution. A comparison of continuous variables was carried out using a Student’s $t$ test, whereas a comparison of non-parametric variables was carried out using a Mann–Whitney test. Statistical analysis of categorical variables was also carried out using $\chi^2$ analysis and Fisher’s exact analysis. Comparisons between the intensive and moderate LLT groups were analysed using a two-way repeated-measure ANOVA and the Kruskal–Wallis test for non-normally distributed variables. Pearson’s correlation coefficients were used to examine the relationship between changes in EPC and lipid profiles. A value of $P < 0.05$ was considered statistically significant.

**RESULTS**

**Baseline and clinical characteristics of study population**

Baseline and clinical characteristics of the study populations are shown in Table 1. There was no significant difference between the CAD and control groups with regard to age or gender. Furthermore, there were no significant differences in the baseline and clinical characteristics between the intensive and moderate LLT groups (Table 1).

**EPC counts, RTL and oxidative DNA damage at baseline**

Results of the EPC analysis in the CAD and control groups are shown in Table 1. The number of circulating CD34/KDR-positive cells and DiI-labelled acLDL/UEA-1-positive cells were lower in the CAD group than in the control group. The RTL of EPCs was significantly lower in the CAD group than in the control group. The RTL did not differ between freshly isolated PBMCs and cultured EPCs in the two groups (RTL in PBMCs, 49.1 ± 11.2 % in the CAD group and 72.9 ± 10.3 % in the control group; both $P > 0.05$ compared with the RTL in EPCs). In addition, 8-hydroxyl 2′-deoxyguanosine MFI of EPCs was higher in the CAD group compared with the control group. There was no significant difference in the number of circulating CD34/KDR-double-positive cells between the intensive and moderate LLT groups. After 8 days of culture, there was no difference in the number of DiI-labelled acLDL/UEA-1-double-positive cells between the two groups. In addition, RTL and 8-hydroxyl 2′-deoxyguanosine MFI of EPCs did not differ between the two groups.

**Lipid profiles after 12 months of statin therapy**

As shown in Table 2, there was no significant difference in baseline cholesterol levels between the intensive and moderate LLT groups. Both groups had decreased total cholesterol and LDL-cholesterol levels, and increased HDL (high-density lipoprotein)-cholesterol levels in CAD patients after 12 months of therapy. Decreases in total cholesterol and LDL-cholesterol levels after 12 months of therapy were significantly greater in the intensive LLT group than in the moderate LLT (0.70 ± 0.10- compared with 0.83 ± 0.10-fold change in total cholesterol respectively; 0.57 ± 0.13- compared with 0.70 ± 0.20-fold change in LDL-cholesterol respectively; both $P < 0.01$). In addition, there was a greater increase in
Table 1 Baseline and clinical characteristics of study population

*P < 0.05 compared with the CAD group. ACEi, angiotensin-converting enzyme inhibitors; ARBs, angiotensin II type 1 receptor blockers; BMI, body mass index; circulating EPCs, CD34/KDR-double-positive cells (per 10⁶ events); cultured EPCs, Dil-labelled acLDL/UEA-1-double-positive cells (cells/mm²); DBP, diastolic blood pressure; N/A, not available; oxidative DNA damage, 8-hydroxyl 2′-deoxyguanosine MFI; SBP, systolic blood pressure.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Total (n = 100)</th>
<th>Intensive LLT group (n = 50)</th>
<th>Moderate LLT group (n = 50)</th>
<th>Control (n = 25)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>64.9 ± 10.1</td>
<td>64.6 ± 10.6</td>
<td>65.3 ± 9.7</td>
<td>65.1 ± 9.5</td>
</tr>
<tr>
<td>Male gender (n)</td>
<td>80 (80 %)</td>
<td>40 (80 %)</td>
<td>40 (80 %)</td>
<td>20 (80 %)</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>25.8 ± 3.5</td>
<td>26.4 ± 4.1</td>
<td>25.3 ± 2.7</td>
<td>24.0 ± 4.2*</td>
</tr>
<tr>
<td>Hypertension (n)</td>
<td>65 (65 %)</td>
<td>32 (64 %)</td>
<td>33 (66 %)</td>
<td>10 (40 %)*</td>
</tr>
<tr>
<td>Diabetes mellitus (n)</td>
<td>27 (27 %)</td>
<td>7 (14 %)</td>
<td>9 (18 %)</td>
<td>0%</td>
</tr>
<tr>
<td>Previous angina (n)</td>
<td>16 (16 %)</td>
<td>7 (14 %)</td>
<td>9 (18 %)</td>
<td>0%</td>
</tr>
<tr>
<td>Smoking (n)</td>
<td>39 (39 %)</td>
<td>21 (42 %)</td>
<td>21 (42 %)</td>
<td>6 (24 %)*</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>126.1 ± 13.8</td>
<td>126.0 ± 14.1</td>
<td>127.6 ± 11.6</td>
<td>121.9 ± 14.6</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>69.8 ± 9.6</td>
<td>71.1 ± 9.5</td>
<td>68.4 ± 9.5</td>
<td>70.8 ± 10.2</td>
</tr>
<tr>
<td>Medication (n)</td>
<td>N/A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aspirin</td>
<td>100 (100 %)</td>
<td>50 (100 %)</td>
<td>50 (100 %)</td>
<td></td>
</tr>
<tr>
<td>ACEi/ARBS</td>
<td>60 (60 %)</td>
<td>31 (62 %)</td>
<td>29 (58 %)</td>
<td></td>
</tr>
<tr>
<td>β-Blockers</td>
<td>40 (40 %)</td>
<td>19 (38 %)</td>
<td>21 (42 %)</td>
<td></td>
</tr>
<tr>
<td>Calcium antagonists</td>
<td>41 (41 %)</td>
<td>20 (40 %)</td>
<td>21 (42 %)</td>
<td></td>
</tr>
<tr>
<td>Nitrates</td>
<td>45 (45 %)</td>
<td>22 (44 %)</td>
<td>23 (46 %)</td>
<td></td>
</tr>
</tbody>
</table>

| Number of EPCs                   |                |                             |                            |                  |
| Circulating EPCs                 | 21.2 ± 6.3     | 21.5 ± 6.3                  | 21.0 ± 6.3                 | 25.4 ± 6.2*      |
| Cultured EPCs                    | 77.6 ± 23.2    | 79.1 ± 19.1                 | 76.1 ± 28.8                | 104.3 ± 36.9*    |
| EPC telomere length              | 48.8 ± 18.2    | 48.8 ± 19.3                 | 48.8 ± 17.2                | 71.0 ± 21.8*     |
| Oxidative DNA damage             | 261.5 ± 110.5  | 256.1 ± 106.0               | 264.8 ± 115.5              | 159.9 ± 58.7*    |

Table 2 Lipidaemic profile of CAD patients treated with statin therapy

*P < 0.05 compared with baseline; †P < 0.05 compared with the moderate LLT group (12 months).

<table>
<thead>
<tr>
<th>Lipid</th>
<th>Intensive LLT group (n = 50)</th>
<th>Moderate LLT group (n = 50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid</td>
<td>Baseline 12 months</td>
<td>Baseline 12 months</td>
</tr>
<tr>
<td>Total cholesterol (mg/dl)</td>
<td>255.3 ± 23.8</td>
<td>177.0 ± 22.7†</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>48.5 ± 11.2</td>
<td>61.8 ± 18.0†</td>
</tr>
<tr>
<td>LDL-cholesterol (mg/dl)</td>
<td>158.3 ± 27.9</td>
<td>98.9 ± 27.1†</td>
</tr>
</tbody>
</table>

HDL-cholesterol levels in the intensive LLT group than in the moderate LLT group (1.34 ± 0.48-fold change in HDL-cholesterol; P = 0.03).

Effect of statin therapy on EPCs

As shown in Figure 1, compared with with baseline, 12 months of intensive LLT markedly increased the number of circulating CD34/KDR-double-positive cells (21.5 ± 6.3 compared with 24.0 ± 6.7 per 10⁶ events respectively; P < 0.01; Figure 1C) and Dil-labelled acLDL/UEA-1-double-positive cells (79.1 ± 19.1 compared with 100.5 ± 33.4 cells/mm² respectively; P < 0.01; Figure 1E), whereas moderate LLT did not change the number of circulating CD34/KDR-double-positive cells (21.0 ± 6.3 compared with 21.8 ± 6.2 per 10⁶ events respectively; P value was not significant; Figure 1D) or Dil-labelled acLDL/UEA-1-double-positive cells (76.1 ± 26.8 compared with 79.9 ± 26.3 cells/mm² respectively; P value was not significant; Figure 1F). As shown in Figure 2, compared with with baseline, 12 months of intensive LLT did not change the RTL (48.8 ± 19.3 compared with 48.8 ± 19.0 respectively; P value was not significant; Figure 2E), but decreased 8-hydroxyl 2′-deoxyguanosine MFI (256.1 ± 106.0 compared with 224.4 ± 109.9 respectively; P < 0.01; Figure 2G). However, compared with with baseline, 12 months of moderate LLT decreased the RTL of EPCs (48.8 ± 17.2 compared with 46.9 ± 16.8 respectively;
Figure 1  Effect of intensive and moderate LLT on the number of EPCs in patients with CAD
Representative examples of the FACS analysis are shown in (A) and (B). Intensive LLT significantly increased the number of CD34/KDR-positive cells (A and C) and Dil-labelled acLDL/UEA-1-double-positive EPCs (E). Moderate LLT did not increase the number of CD34/KDR-positive cells (B and D) or Dil-labelled acLDL/UEA-1-positive EPCs (F). ns, not significant.

$P < 0.05$; Figure 2F), but did not change 8-hydroxyl 2'-deoxyguanosine MFI ($266.8 \pm 115.5$ compared with $260.1 \pm 112.5$ respectively; $P$ value was not significant; Figure 2H).

**Correlation between changes in EPCs and LDL-cholesterol levels after statin therapy**
There was a weak negative correlation between changes in EPC number and changes in LDL-cholesterol levels after intensive LLT ($r = -0.37, P < 0.01$ for circulating EPC counts compared with LDL-cholesterol levels; $r = -0.38, P < 0.01$ for cultured EPC counts compared with LDL-cholesterol levels), but not after moderate LLT ($r = -0.11, P = 0.47$ for circulating EPC counts compared with LDL-cholesterol levels; $r = -0.26, P = 0.07$ for cultured EPC counts compared with LDL-cholesterol levels). There was a weak positive correlation between changes in oxidative DNA damage and LDL-cholesterol levels after intensive LLT ($r = 0.30, P < 0.05$), but not after moderate LLT ($r = -0.05, P = 0.75$). In contrast, changes in the RTL of EPCs were weakly negatively correlated with changes in LDL-cholesterol levels after moderate LLT ($r = -0.60, P < 0.01$), but not after intensive LLT ($r = -0.03, P = 0.85$).

**In vitro study with statin treatment**
The RTL decreased in t-BHP- or BSO-stimulated EPCs (Figure 3). The percentage changes in telomere length...
were lower in cultured EPCs treated with atorvastatin and pravastatin than in those with vehicle (Figure 3). Percentage changes in telomere length were lower in cultured EPCs treated with atorvastatin compared with those with pravastatin. The inhibition of telomere shortening in cultured EPCs treated with atorvastatin was dose-dependent, with a maximal inhibitory effect achieved at 0.5 μmol/l.

**DISCUSSION**

The main findings of the present study are: (i) EPC telomere length was significantly shorter in the CAD group than in the control group; (ii) intensive LLT increased EPC numbers and prevented EPC telomere shortening, whereas moderate LLT did not; (iii) changes in EPC numbers and telomere length were related to changes in LDL-cholesterol levels after statin therapy; and (iv) treatment with atorvastatin affected the inhibition of EPC telomere shortening compared with pravastatin in an *in vitro* study.

Endothelial cell injury is regarded as the classical stimulus for the development of atherosclerotic lesions [17]. It has been reported that endothelial injury in the absence of sufficient circulating EPCs may affect the progression of cardiovascular disease [18]. An autopsy study [19] has also shown that the telomere length of coronary

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*Figure 2  Effect of intensive and moderate LLT on RTL and 8-hydroxyl 2′-deoxyguanosine MFI of EPCs in patients with CAD*

Representative examples are shown in (A–D). Intensive LLT did not change RTL (A and E), but significantly decreased 8-hydroxyl 2′-deoxyguanosine MFI (C and G), whereas moderate LLT shortened RTL (B and F), but did not change 8-hydroxyl 2′-deoxyguanosine MFI (D and H). *ns*, not significant.
endothelial cells was markedly shorter in CAD patients than in non-CAD patients. The present study has shown telomere shortening and increased oxidative DNA damage in EPCs from CAD subjects compared with controls. To exclude telomere shortening as a cause of premature senescence in EPCs, we measured the RTL in PBMCs from individual subjects. The RTL did not vary between PBMCs and EPCs, thus eliminating culture-induced telomere erosion as a probable cause of senescence in EPCs. These observations suggest that EPC telomere erosion may accelerate the rate of senescence in coronary endothelial cells and may contribute to the progression of coronary atherosclerosis.

A clinical trial [12] using an intravascular ultrasound method for imaging the coronary vessel wall (measurement of atheroma burden) showed reduced progression of coronary atheroma burden with intensive LLT with atorvastatin, whereas moderate LLT with pravastatin had an increase in coronary atheroma burden in CAD patients. That study suggests that intensive LLT with atorvastatin reduces the progression of coronary atherosclerosis compared with moderate LLT with pravastatin. Endothelial cells with senescence phenotypes are found in human atherosclerotic plaques, suggesting that endothelial cell aging may contribute to atherogenesis [20,21]. In the present study, intensive LLT resulted in increases in the numbers of EPCs and prevented EPC telomere shortening compared with moderate LLT. Our present in vitro study has also demonstrated that oxidative stress significantly diminished telomere length, an effect that was abolished by treatment with atorvastatin compared with pravastatin. There were differential effects of statins between the in vivo and in vitro studies. Treatment with pravastatin did not prevent EPC telomere shortening, although cultured EPCs treated with pravastatin did inhibit telomere shortening compared with vehicle. This can be explained by the difference in statin dose between the in vivo and in vitro studies, therefore the biological effect of statin treatment in CAD patients may have been lower than in the in vitro study. Low-dose t-BHP and BSO treatment induces intracellular oxidative stress via the glutathione redox cycle, but has no cytotoxic or cytostatic effects [22]. It is well-known that increased oxidative stress is a key step in the initiation and progression of atherosclerosis [23]. An in vitro model using cultured human endothelial cells has shown that low-dose atorvastatin prevents endothelial cell senescence via an atorvastatin-mediated decrease in ROS (reactive oxygen species) production [24]. This may be, at least in part, explained by differences in lipophilicity, because atorvastatin is more lipophilic and would therefore be more likely to permeate through cell membranes than pravastatin [25]. The present study has shown that treatment with atorvastatin decreased oxidative DNA damage in EPCs compared with treatment with pravastatin. In agreement with the findings of the present study, an active metabolite of atorvastatin, but not pravastatin, inhibited membrane cholesterol domain formation as a function of oxidative stress, suggesting that atorvastatin may have the most antioxidant properties among the statins [25]. These observations would suggest that intensive LLT may have a preventive effect on EPC-telomere erosion-induced senescence through its antioxidant effects.

The present study has also shown that LDL-cholesterol levels were significantly decreased by intensive LLT compared with moderate LLT.
in LDL-cholesterol levels was related to an increase in EPC number and a decrease in oxidative DNA damage in the intensive LLT group. On the other hand, intensive LLT did not change EPC telomere length. A recent study has reported that LDL-cholesterol is an independent predictor of reduced numbers of circulating EPCs in essential hypertension patients [26]. These observations therefore suggest that intensive LLT may increase EPC proliferation and prevent EPC telomere shortening.

A limitation of the present study is the small number of CAD patients receiving each statin therapy. The EPC study was performed using cultured EPC samples rather than isolated EPCs, because the number of isolated EPCs is low (10–30 per 1×10⁶ cells), meaning isolated cells are not useful for EPC study. Most of the results of the present study may therefore have been influenced by an in vitro effect after culturing of EPCs. The present study used a relatively low dose of statin (10 mg/day) compared with other reports, because this dose of statins is the most commonly prescribed dose for Japanese patients. The present study also used two different statins (lipophilic and hydrophilic statins) for comparison because we did not expect a large difference in the effects on EPC analysis when comparing the same statin at different dosages. Therefore further studies will therefore be needed to determine whether there is a causal relationship between EPC telomere erosion and vulnerable coronary plaques.

In conclusion, our present findings suggest that intensive LLT may prevent EPC telomere erosion in patients with CAD, possibly contributing to the beneficial effects of intensive LLT in this disorder. Therefore further studies are required to examine the mechanism underlying the effects of intensive LLT using a statin on EPC telomere biology in CAD.

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