Vascular Smooth Muscle Cells Undergo Telomere-Based Senescence in Human Atherosclerosis
Effects of Telomerase and Oxidative Stress

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Abstract—Although human atherosclerosis is associated with aging, direct evidence of cellular senescence and the mechanism of senescence in vascular smooth muscle cells (VSMCs) in atherosclerotic plaques is lacking. We examined normal vessels and plaques by histochemistry, Southern blotting, and fluorescence in situ hybridization for telomere signals. VSMCs in fibrous caps expressed markers of senescence (senescence-associated β-galactosidase [SAβG] and the cyclin-dependent kinase inhibitors [cdkis] p16 and p21) not seen in normal vessels. In matched samples from the same individual, plaques demonstrated markedly shorter telomeres than normal vessels. Fibrous cap VSMCs exhibited markedly shorter telomeres compared with normal medial VSMCs. Telomere shortening was closely associated with increasing severity of atherosclerosis. In vitro, plaque VSMCs demonstrated morphological features of senescence, increased SAβG expression, reduced proliferation, and premature senescence. VSMC senescence was mediated by changes in cyclins D/E, p16, p21, and pRB, and plaque VSMCs could reenter the cell cycle by hyperphosphorylating pRB. Both plaque and normal VSMCs expressed low levels of telomerase. However, telomerase expression alone rescued plaque VSMC senescence despite short telomeres, normalizing the cdki/pRB changes. In vivo, plaque VSMCs exhibited oxidative DNA damage, suggesting that telomere damage may be induced by oxidant stress. Furthermore, oxidants induced premature senescence in vitro, with accelerated telomere shortening and reduced telomerase activity. We conclude that human atherosclerosis is characterized by senescence of VSMCs, accelerated by oxidative stress-induced DNA damage, inhibition of telomerase and marked telomere shortening. Prevention of cellular senescence may be a novel therapeutic target in atherosclerosis. (Circ Res. 2006;99:156-164.)

Key Words: atherosclerosis ■ smooth muscle ■ aging

H uman atherosclerotic plaques comprise inflammatory cells (macrophages and T lymphocytes), vascular smooth muscle cells (VSMCs), and intracellular and extracellular lipids. Plaque disruption results in acute myocardial infarction and stroke, whereas repeated rounds of subclinical rupture and repair also promote plaque growth. Although VSMC proliferation occurs in atherogenesis, most proliferating cells are macrophages, and VSMC mitotic rates are lower in advanced plaques than early lesions, even after plaque rupture,1 suggesting that plaque VSMCs may exhibit senescence.

Cellular senescence can be defined as cell cycle arrest accompanying the exhaustion of replicative potential.2 Senescent cells display a characteristic morphology (vacuolated, flattened cells) and gene expression, including markers such as senescence-associated β-galactosidase (SAβG).3 Senescence may be triggered by 2 broadly different mechanisms. In most primary cells, the telomeres of chromosomes shorten at each cell division because of incomplete chromosomal replication. Replicative senescence may be induced at critical telomere lengths or structures, such as telomeric fusion or dicentrics or loss of telomere-bound factors.4,5 Cells also undergo “stress-induced premature senescence” (SIPS), for example, in response to activated oncogenes (eg, Ha-Ras) and suboptimal culture conditions.6

Although telomere loss occurs with replication, both premature senescence and telomere breaks may be induced by oxidative DNA damage. Reactive oxygen species (ROS), particularly superoxide anions, hydrogen peroxide, and hydroxyl radicals, can produce a large variety of DNA damage, including DNA strand breaks and DNA base modifications. ROS can accelerate telomere loss during replication in some cell types7 but also induces premature senescence independently of telomere shortening.8 Increased levels of ROS are
found in atherosclerosis in all layers of the diseased arterial wall and, particularly, in the plaque itself. These findings suggest that ROS within plaques may promote cellular senescence.

Reproductive senescence and SIPS converge on the tumor suppressor genes p53 and rb, the latter being regulated by cyclin-dependent kinase inhibitors (cdkis), including p16 and p21. pRB, p53, p21, or p16 expression can induce senescence and are often increased in both reproductive senescence and SIPS. Telomere loss or damage activates p53 via DNA damage-sensing mechanisms, with subsequent transcription of p21, whereas stress-induced activation of p16 accelerates replicative senescence independently of telomere length. In addition, p16 expression effectively renders telomere-based senescence irreversible, in part, by promoting repressive heterochromatin at loci containing targets of E2F transcription factors. Although the division between replicative senescence and SIPS is useful, the pathways have multiple areas of overlap. Indeed, both telomere-based DNA damage and stress-induced activation of p16 may occur simultaneously, inducing a growth arrest with cell cycle regulator expression, reflecting activation of both pathways.

We therefore examined both the evidence for senescence in atherosclerosis and the mechanism of growth arrest in human plaque VSMCs. We demonstrate that plaque VSMCs show multiple markers of senescence in vivo and in vitro and demonstrate marked telomere shortening. We further show that plaque VSMC senescence is completely rescued by telomerase but accelerated by oxidative stress.

Materials and Methods
Isolation of VSMCs From Human Tissue
Normal human aortic medial vascular smooth muscle cells (VSMCs) were isolated from recipients undergoing cardiac transplant (n=10) and plaque-derived VSMCs from carotid atherectomies (n=50) with informed consent using protocols approved by the Cambridge or Huntingdon Research Ethical Committee. VSMCs were isolated, cultured, and characterized as previously described using immunofluorescence labeling for α-smooth muscle actin (α-SMA), sm muscle myosin, calponin, desmin, and vimentin (Figure I in the online data supplement, available at http://circres.ahajournals.org). Contamination with other cell types was excluded using markers for ECs, macrophages, and T lymphocytes. Separate cultures isolated from individual patients were studied. Cells were passaged at 80% confluence and split 1:2. Proliferation rates were determined for cells at approximately 50% confluence as previously described.

Coronary endarterectomy plaque and normal artery segments (aorta and bypass conduit) were isolated at coronary artery bypass surgery and coronary artery segments from patients undergoing cardiac transplantation obtained from recipient hearts.

Measurement of Telomere Length
See the online data supplement.

Telomere/Immunostaining Fluorescence In Situ Hybridization
Telomere/immunostaining fluorescence in situ hybridization (TELI-FISH) was performed using a modification of previous methods. Immuno staining and immunohistochemistry

SAβG Staining and Immunohistochemistry
See the online data supplement.

Immunoblotting and Immunoprecipitation
Immunoblotting and immunoprecipitation were performed as previously described.

Virus-Based Expression of hTERT, Cyclin D1, and CDK4
Retroviruses were used to infect VSMCs as previously described. Adenoviruses encoding cyclin D1 and CDK4 were used to express these products in human plaques VSMCs (see the online data supplement).

Real-Time Quantitative Telomeric Repeat Amplification Protocol Assay for Telomerase Activity
Telomerase activity was measured by real-time quantitative telomeric repeat amplification protocol (RTQ-TRAP) assay as previously described (see the online data supplement).
Determination of Mitochondrial Mass and ROS Levels

The fluorescent dyes H$_2$DCFDA and acridine orange (Molecular Probes) were used to measure cellular ROS content and mitochondrial mass, respectively. (See the online data supplement).

Statistics

An unpaired 2-tailed Student’s t test was used to examine differences between 2 groups, when data approximated a normal distribution. When data did not approximate a normal distribution, Mann–Whitney U test was used.

Results

Evidence for VSMC Senescence in Atherosclerosis

We first examined expression of senescence markers in plaque VSMCs in vivo including SAβG, coexpression of the cdkis p16/p21/p27 with SAβG, and cell-lineage markers in human carotid atherectomies (Figure 1). Among fibrous cap VSMCs, 18% ± 2.6 (mean ± SEM, n = 6) were SAβG positive, but SAβG-positive VSMCs were not found in normal vessels (n = 6). Macrophages showed faint SAβG expression throughout plaques, most likely reflecting their high lysosomal content, but < 5% of lymphocytes were SAβG positive. More than 70% SAβG-positive VSMCs also expressed p16, and > 90% expressed p21, although neither p16 nor p21 was expressed in normal vessels. p27 expression was evident in both normal medial and plaque VSMCs and correlated poorly with SAβG, suggesting a limited role for p27 in plaque VSMC senescence.

We next examined expression of these markers in cultured plaque or normal VSMCs. First passage plaque VSMCs demonstrated 80.5% ± 12.0 (mean ± SEM) SAβG expression compared with 5.2% ± 1.7 (mean ± SEM, P < 0.01) in normal VSMCs. Both VSMC types reached near 100% expression over time, at passage 3 to 4 for plaque VSMCs and 10 to 12 for normal VSMCs. Plaque VSMCs exhibited large, flattened morphology and low cell density, with lower rates of cell proliferation on time-lapse videomicroscopy at any time point (eg, median [interquartile range]: 19.64 [15.4 to 25.2] versus 65.98 [46.2 to 92.6] divisions per 100 cells per 24 hours [U = 4, P < 0.001] at passage 3). Plaque VSMCs underwent culture senescence at passage ≥ 3 compared with passage ≥ 8 in normal VSMCs.

Cell Cycle Regulation Is Disrupted in Human Plaque VSMCs

Expression of G1 regulatory proteins in plaque and normal VSMCs was examined at passage 1 to 2, when marked differences in cell proliferation were observed. Plaque VSMCs demonstrated higher levels of nonphosphorylated pRB, reduced cyclin D1,3, reduced cyclin E and p27, but increased p16 and p21 (Figure 2A). No differences were observed in CDK2 or CDK4. Plaque VSMCs had reduced E2F-1 expression and a higher proportion of E2F-1 bound to pRB. E2F-2 and E2F-3 levels were unchanged. To determine whether the pattern of pRB, p21, and p16 expression was unique to plaque VSMCs, we examined their expression at replicative senescence of normal VSMCs (Figure 2B). p16 was elevated with reduced cyclins D and E and pRB phosphorylation, although p21 expression was unchanged. Thus, senescence of plaque VSMCs may be attributable to other pathways interacting with replication arrest.

To prove that pRB phosphorylation is required for plaque VSMC senescence, we expressed CDK4 and cyclin D1 in human plaque VSMCs using adenovirus vectors and examined cell cycle progression by 5-bromodeoxyuridine (BrdU) incorporation and protein expression by Western blot (Figure 3). CDK4 and cyclin D1 expression induced robust phosphorylation of pRB, accompanied by increased expression of E2F-1 and the G2/M cyclin, cyclin A. pRB phosphorylation was sufficient to induce cell cycle entry of plaque VSMCs, as demonstrated by increased BrdUrd incorporation (Figure 3).

Telomere Length Is Markedly Shorter in Plaque VSMCs

To examine whether the premature senescence of plaque VSMCs reflected telomere loss, we first examined telomere restriction fragment (TRF) length in cultured normal aortic VSMCs. VSMC telomeres progressively shortened with increasing passage, by ~ 100 bp per cumulative population doubling (CPD) (Figure 4A). To examine TRF length in vivo, we isolated coronary artery segments from 20 patients undergoing cardiac transplantation for ischemic or dilated cardiomyopathy, aged between 40 to 60 years; parallel histology demonstrated advanced atherosclerosis or no atherosclerosis, respectively. Despite similar chronological age, TRF length exhibited large variations between individuals in either group (Figure 4B). To circumvent this variability, we studied matched diseased and normal vessels from the same patient, using coronary endarterectomies and bypass conduits (left internal mammary artery, aorta, radial artery). Normal vessels from different vascular beds in the same patient showed identical telomere length (Figure 4C), allowing comparison of normal and atherosclerotic arteries from different sites. Coronary plaque telomeres were ~ 1 kb (range 0.74 to 1.31) (P < 0.01) shorter than matched normal vessels (Figure 4D).
telomere loss in vivo occurs to the same extent per division to telomere loss in vitro, and is confined to VSMCs, this difference represents approximately 7 to 13 CPDs.

Plaques are heterogeneous structures, containing cells not present in normal arteries. To examine VSMC telomere lengths, we used quantitative TELI-FISH (qTELI-FISH), a semiquantitative assay based on binding of fluorescently labeled peptide nucleic acid complexes complementary to telomere sequences. We examined plaques with a range of disease severity (American Heart Association Grades I through V)\(^8\) for telomere length, double-labeled with α-SMA to identify VSMC telomere signals in the fibrous cap (Figure 5A), with VSMCs in the remote, uninvolved arterial media acting as internal controls. Telomere signals were easily detected in α-SMA negative cells in the plaque and in medial VSMCs. In contrast, both telomere intensity and average telomere number were markedly reduced in intimal VSMCs (Figure 5B and 5C). Total telomere fluorescence (number of telomeres \(\times\) fluorescence intensity of each telomere) of medial VSMCs did not change significantly with increasing disease severity. In contrast, telomere signals were significantly reduced in intimal plaque VSMCs versus medial VSMCs in the same section (Figure 5C) and were inversely proportional to disease severity, with signals barely detectable in the most advanced lesions.

**Telomerase Delays VSMC Senescence**

Although telomerase enzymatic activity requires TERT, TERC, and other proteins, hTERT expression is a rate-limiting factor in telomerase activity. We therefore examined telomerase (hTERT) expression in plaques or normal vessels. Telomerase expression was low in both human plaque and normal vessel VSMCs in vivo (Figure 6A through 6C). In contrast, telomerase expression was easily detectable in macrophages (Figure 6D).

To examine whether telomere maintenance could block VSMC senescence, we stably reexpressed hTERT or vector control in human VSMCs using retrovirus vectors. Antibiotic selection was used to produce cultures whereby all cells expressed the product. Parallel cultures were maintained in identical culture conditions and passaging. Normal VSMCs expressing the vector showed little telomerase expression on Western blotting; activity measured by RTQ-TRAP, a highly sensitive and quantitative assay for telomerase activity,\(^9\) was barely above background (heated controls). hTERT VSMCs expressed detectable telomerase (Figure 6E) and significantly elevated telomerase activity (0.202±0.011 arbitrary units versus 0.06±0.006 in control VSMCs [n=3 mean±SEM]). VSMCs expressing the vector senesced around passage 15, expressing p16 and low levels of phosphorylated pRB, cyclin D\(_{1,3}\) and cyclin E. hTERT delayed senescence of normal VSMCs by >30 CPDs and effectively immortalized the cultures. This was associated with maintained cyclins D\(_{1,3}\) and E expression and pRB phosphorylation despite high levels of p21 and p16, indicating that ongoing proliferation occurred despite normal arrest signals associated with senescence. hTERT expression significantly elevated telomerase activity in plaque VSMCs (0.26±0.006 versus 0.029±0.005 in non-infected plaque cells). Plaque VSMCs expressing the control vector do not survive selection, as cells undergo senescence before a culture can be established. In contrast, hTERT rescued plaque VSMC senescence, completely reversed the high p21 and p16 expression seen in control plaque VSMCs, and normalized P-pRB despite having minimal effects on cyclin D\(_{1,3}\) and cyclin E (Figure 6E). Importantly, hTERT did not reduce TRF loss/CPD in normal VSMCs, and hTERT VSMCs showed similar TRF lengths at the CPD that vector- VSMCs underwent senescence (Figure 6F and 6G). However, hTERT allowed cells to proliferate at telomere lengths far shorter than those seen at VSMC senescence.

**Oxidative DNA Damage Accelerates Telomere Loss and Induces Early Senescence**

To examine the role of oxidative stress in VSMC senescence in atherosclerosis, we first examined plaques for 8-oxo-G, an abundant oxidative lesion in mammalian DNA.\(^8\) 8-Oxo-G was not detected in medial VSMCs of normal arteries or uninvolved segments of arteries containing plaques. In contrast, 22%±4.8 of fibrous cap VSMCs and 35%±6.7
We next examined oxidative stress in plaque and normal VSMCs, in normal VSMCs at replicative senescence, and after treatment of VSMCs with tert-butylhydroperoxide (t-BHP), an inducer of hydrogen peroxide \(^2\) that can induce DNA single-strand breaks.\(^2\) Flow cytometric analysis of fluorescence of the redox-sensitive dye H₂-DCFDA showed significantly increased oxidative stress in plaque versus normal VSMCs and also in normal VSMCs at replicative senescence. These changes paralleled a significant increase in mitochondrial mass in plaque and senescent VSMCs (supplemental Figure II). t-BHP also significantly induced oxidative stress (supplemental Figure II). A single (acute) administration of 80 mmol/L t-BHP for 1 hour induced growth arrest (13.2±1.4% versus 2.8±1.0% Ki67-positive cells, \(P<0.01\)) and increased the percentage of SAβG from 11.9±1.3% to 55.5±0.4% (\(P<0.01\) [mean±SEM]), without inducing cell death, as determined by time-lapse videomicroscopy and trypan blue exclusion (not shown). To mimic chronic oxidant stress, human VSMCs were treated every passage with t-BHP (16 to 80 mmol/L) for 1 hour, then cells returned to medium without t-BHP. Cell proliferation, passage number at senescence, telomere loss with each CPD, and G₁/S regulator expression were examined, compared with untreated controls. Chronic t-BHP treatment dose-dependently accelerated senescence and increased telomere loss/CPD (Figure 7E and 7F). t-BHP induced p21, and reduced pRB phosphorylation and cyclins D₁,3 and E, although p16 expression was unchanged (Figure 7G). t-BHP also reduced telomerase activity in a dose-dependent manner, even at concentrations that did not accelerate telomere shortening (Figure 7H).

**Discussion**

We identify human atherosclerosis as a disease characterized by VSMC senescence. SAβG-positive VSMCs were readily detected within advanced human plaques, colocalized with p16 and p21, confirming a senescence phenotype in vivo. Previous studies have demonstrated SAβG activity in human plaques, in both ECs and VSMCs.\(^{24,26}\) However, although SAβG is a useful marker, its activity is critically dependent on detection conditions, and it is also expressed in nonsenescent cells with a high lysosomal content (Kurz et al\(^1\) and shown here). Multiple markers of senescence are therefore recommended to demonstrate senescence in vivo, together with cell lineage markers, to identify cell type undergoing senescence. Although there were minor differences in expression of specific markers in vitro and in vivo, mostly likely reflecting culture conditions, atherosclerotic plaque VSMCs in vitro manifest characteristic morphological features of senescence, impaired proliferation and early culture senescence, and express multiple senescence markers (SAβG, p21, p16). Increased p16 and p21 coupled with low cyclin D and E expression lead to reduced pRB phosphorylation and increased E2F-1 bound to pRB, preventing E2F-1–mediated gene activation required for S-phase progression. We show
that impaired pRB phosphorylation induces plaque VSMC senescence, as pRB phosphorylation (by ectopic CDK4 and cyclin D1 expression) is sufficient to induce plaque VSMC cell cycle transition. We show that p16 (but not p21) expression occurs in replicative senescence of normal VSMCs in culture, whereas p21 (but not p16) occurs after oxidative stress. Our data therefore suggest that plaque VSMC senescence is attributable to a combination of (oxidative) DNA damage accelerating replicative senescence, thereby activating both p21 and p16 and inducing pRB hypophosphorylation (Figure 8).

To determine whether atherosclerosis is accompanied by telomere shortening in vivo, we examined matched normal arteries and plaques from the same patient. Telomeres in whole human plaques were markedly shorter than in matched normal vessels, by approximately 1 kb in advanced lesions. If only VSMCs have shortened telomeres, this represents at least 7 to 13 additional CPDs. Although ECs and peripheral leukocytes may show telomere attrition in atherosclerosis, this calculation may underestimate VSMC telomere loss, as non-VSMCs retained telomere signals even in advanced lesions (Figure 5). Intimal VSMC telomere signals were inversely correlated with disease status, with even early lesions associated with significant telomere shortening.

Previous studies have found that age-dependent telomere attrition is higher in both intima and media of the distal versus proximal abdominal aorta, the intima of iliac artery versus internal thoracic artery, or endothelial cells from plaque versus normal arteries. However, the first 2 studies did not identify which cells showed telomere loss, and VSMCs have not been studied previously. In addition, any relationship between telomere length and atherosclerosis was lost after adjustment for age. In contrast, using qTEL-FISH to provide the first detailed mapping of telomere signals in atherosclerosis, we show that plaque VSMCs within the fibrous cap undergo marked telomere loss compared with medial VSMCs of the same lesion. Telomere signals are barely detectable in advanced plaque VSMCs, whereas non-VSMCs demonstrate robust signals. Importantly, it is relative rather than absolute telomere length that correlates with plaque development. Plaques at the same stage of development show similar telomere loss compared with matched normal vessels, despite marked heterogeneity of telomere length in normal vessels or plaques between individuals of the same chronological age.

The telomere loss in plaque VSMCs most likely represents additional replications involved in lesion development.
VSMCs of fibrous caps in plaques are monoclonal in origin,31,32 a phenomenon not seen in ECs or inflammatory cells.32 Clonal expansion may represent proliferation in a developmentally determined “patch” of intimal cells, selective recruitment of VSMCs of a specific phenotype, or genetic alteration in a small number of cells. Although our data cannot determine which of these possibilities is occurring, the 7 to 13 CPD required for 1-kb telomere loss favors selective expansion of a small number of cells. The telomere loss in intimal VSMCs of early (type I and type II) lesions, which demonstrate the highest rates of cell proliferation, suggests that such selection occurs early in atherosclerosis. Apoptosis of VSMCs, which is also a feature of advanced atherosclerosis, could also promote telomere shortening by reducing the number of cells left in the cap that can replicate.

We find that oxidative stress accelerates telomere shortening in vitro, suggesting that oxidative DNA damage in plaques in vivo may accelerate telomere shortening with each replication. ROS (particularly hydroxyl radicals) damage DNA, forming a series of adducts including 8-oxo-G; senescent cells also produce high levels of ROS and contain higher levels of oxidatively damaged DNA.33 We find that 8-oxo-G is expressed highly in both macrophages and VSMCs in plaques, and oxidative stress is increased in plaque and senescent VSMCs. Whereas acute oxidative stress induces growth arrest, chronic stress accelerates senescence and increases telomere loss per CPD. There are numerous mechanisms by which ROS can promote telomere damage or loss. Increased telomere loss per division can occur in individual cells because of a telomere-specific deficiency in base excision repair, leading to preferential accumulation of ROS-induced single-stranded DNA breaks preventing replication of distal telomeres when cells divide. ROS also promote the nuclear export of telomerase in ECs, reducing telomerase activity and promoting senescence.35,36 Although this mechanism has not been shown in VSMCs, we find that ROS inhibit telomerase activity in VSMCs in addition to causing...

Figure 7. Effects of oxidative stress on VSMCs. A and B, Immunohistochemistry for 8-oxo-G (brown) and SMA (blue), demonstrating 8-oxo-G–positive VSMCs in plaque fibrous caps (arrows). C and D, Immunohistochemistry for 8-oxo-G (brown) and CD68 (blue), demonstrating 8-oxo-G–positive macrophages in plaque necrotic cores (arrows). B and D, High-power magnifications of areas outlined in A and C, respectively. Scale bar represents 200 μm in A and C; 50 μm in B and D. E, Southern blot for TRF length in VSMCs before treatment (C) or after 1 hour of treatment with t-BHP (80 mmol/L) or control with increasing CPD (indicated). Mean TRF lengths are as marked. F, Graph showing change in telomere length (ΔTL) with CPD for increasing t-BHP concentrations (n=2). G, Western blot for G1/S regulator expression in control VSMCs (C) or treated with 80 mmol/L t-BHP. H, Telomerase activity (arbitrary units) in normal VSMCs with increasing doses of t-BHP. Means±SEM (n=3).

Figure 8. Model of VSMC senescence in atherosclerosis. Plaque VSMC senescence occurs by a combination of replicative senescence and SIPS. Replication in VSMCs (which have low telomerase activity) induces telomere dysfunction and expression of p16. ROS induce DNA damage in both nuclear and mitochondrial DNA. DNA damage activates a damage response pathway involving activation of p53, with subsequent p21 transcription. p16 and p21 induce pRB hypophosphorylation and senescence. ROS also accelerate telomere loss during replication, in part, by damage to telomeric DNA and reduction in telomerase activity.

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DNA damage. Alternatively, repeated stress increases the proportion of cells undergoing growth arrest, so that only a subset of VSMCs undergoes replication in vivo.

Our data do not exclude the possibility that changes in telomere structure or function or telomerase activity also contribute to atherosclerosis. Similar to previous studies, we find that ectopic hTERT expression prolongs the lifespan of normal VSMCs. Furthermore, telomerase also rescues early plaque VSMC senescence, reversing their typical G1/S regulator pattern. However, in contrast to earlier studies, telomerase maintained VSMC proliferation despite no measurable effect on TRF length, allowing cells to proliferate despite very short telomeres. Low levels of exogenous hTERT can increase proliferative lifespan and reduce chromosome fusions in fibroblasts while telomere length continues to shorten, possibly via actions on chromatin maintenance and DNA damage responses. Telomerase can also promote proliferation independent of its DNA synthesis capacity.

Currently, the role of senescence in atherosclerosis is controversial. Senescence-accelerated mice develop increased atherosclerosis, suggesting that cellular senescence ultimately promotes atherogenesis. In contrast, apoE−/− mice also lacking TERT show less aortic atherosclerosis than control apoE−/− mice, suggesting that TERT deficiency protects against atherogenesis. Although these studies demonstrate that accelerated senescence can affect atherogenesis, the role of senescence in established plaques or specifically in VSMCs was not examined. Both studies resulted in early senescence of all cells, and the effects of TERT deficiency were mostly manifest in inflammatory cells. In humans, VSMC senescence may exert profound effects on atherogenesis and stability of advanced plaques. Most patients with Hutchinson–Gilford progeria syndrome (HGPS), an accelerated aging syndrome, die of atherosclerosis. VSMC depletion is a major feature in progeria, and normal aging, and likely represents replicative senescence, telomere shortening, and decreased capacity for repair, as HGPS VSMCs are more susceptible to hemodynamic and ischemic stress. Replicative senescence and ongoing apoptosis in the fibrous cap would result in cap thinning, frequently seen in advanced human lesions, predisposing to plaque rupture. Senescent cells may also promote plaque instability by overexpressing proteins such as adhesion molecules, regulators of hemeostasis, and matrix metalloproteinases.

In conclusion, we demonstrate that human atherosclerosis is characterized by VSMC senescence and marked telomere shortening and that telomerase expression can delay senescence. Oxidative DNA damage is seen in vivo, and chronic oxidative stress accelerates telomere loss and VSMC senescence. This suggests that human VSMCs undergo a replicative senescence that is accelerated by oxidative stress. Prevention of cellular senescence may be a novel therapeutic target in atherosclerosis.

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Disclosures
None.

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Methods

Immunohistochemistry

Formalin-fixed, paraffin embedded sections were de-waxed in Histoclear and hydrated through graded methanol solutions to water. Primary antibodies were mouse anti-α-smooth muscle actin antibody (1:50, DAKO), anti-CD68 antibody (1:150, DAKO), anti-CD3 antibody (Biocarta), anti-p21 (1:600, Pharmingen), -p16 and -p27 (1:150, Pharmingen), -Telomerase (1:200, Santa Cruz) or -8-oxo-G (1:5000, Japan Institute of Aging) or isotype-matched control. Sections were washed twice in PBS, incubated for 30 minutes in horse anti-mouse biotin-conjugated antibody, washed in PBS and incubated in Avidin-Biotin complex, washed in PBS and incubated with diaminobenzidine (DAB).

For quantification of VSMC expression of SAβG, p21 and p16 in Figure 1, and qTELI-FISH signal in Figure 5, the fibrous cap was defined as the distinct layer of connective tissue completely covering the lipid core on the luminal side, usually consisting of smooth muscle cells in a collagenous-proteoglycan matrix, with varying degrees of infiltration by macrophages and lymphocytes.

Immunoblotting/immunoprecipitation

Infection of human plaque VSMCs with adenoviruses encoding CDK4 and cyclin D₁.

Plaque-derived VSMCs were cultured in slide-flasks. When cells were approximately 70% confluent they were infected with either Rad-empty adenovirus (RAD 60 1600pfu/cell) or a combination of both nuclear localised cyclin D₁ adenovirus (800pfu/cell) and CDK4 adenovirus (800pfu/cell) (generous gifts from Dr Ikeda, Tokyo University). Parallel infection with a β-galactosidase-expressing virus (800pfu/cell) demonstrated that >98% of cells expressed the gene product after infection. After incubation of cells with adenovirus for 24 hours the medium was replaced with fresh HEPES-buffered medium and the cells observed by time lapse videomicroscopy for 72 hours. Protein lysates were taken from these filmed cells and blotted for expression of cyclinD₁, CDK4, pRB, E2F-1and cyclin A as above.

To examine the ability of CDK4 and cyclin D₁ to promote cell cycle entry, plaque-derived VSMCs were cultured in 4-well chamber slides, and cells infected with Rad 60 or both cyclin D₁ and CDK4 adenoviruses. After 2 hours 10µM BrDU was added and cells incubated for a further 22 hours. Cells were then stained for BrDU incorporation by immunocytochemistry.

Measurement of telomere length

High-molecular weight DNA (> 23 Kbp) was isolated from tissues and cultured cells using the DNeasy Kit (Qiagen, UK). DNA quality was assessed by electrophoresis prior to restriction digestion. 4-5 µg DNA was then digested with HinFI and RsaI, separated on a 0.5% TAE agarose gel overnight and transferred to Hybond N+ (Amersham, UK) nylon membrane. Southern blots underwent two rounds of
hybridization, the first performed at 65° C with probes specific to DNA ladders, the second at 37° C with a telomere specific oligonucleotide probe (5’-CCCTAA-3’) labelled with ³²P using PNK (Promega, UK). Radioactive blots and gels were analysed and quantified by phosphorimaging (Packard Cyclone phosphorimager and Optiquant software, Packard Biosciences, USA). Mean telomere length was calculated using the formula: L = ε(ODi)/ε(ODi/Li), where ODi is the integrated signal intensity and Li is DNA length at position i.

**Telomere/Immunostaining-FISH (TELI-FISH)**

Briefly, 7µM tissue sections were deparaffinised in xylene, hydrated in graded ethanol and washed in deionised water containing 0.1% Tween-20. Slides were microwaved in citrate buffer, washed in water and PBST for 5 minutes each, rinsed in deionised water, dehydrated in 95% ethanol and air-dried. 35-40µl Cy3-labelled telomere-specific peptide nucleic acid (PNA, Applied Biosystems, UK). 0.3 µg/ml PNA in 75% formamide, 10mmol/l Tris (pH 7.5), 0.5% Boehringer Mannheim blocking reagent was applied to each slide, the section cover-slipped, slides denatured by incubation at 85° C for 7.5 minutes. Hybridisation was performed in the dark at room temperature for 2h. Coverslips were removed and slides washed twice in PNA wash solution (70 % formamide, 10mmol/l Tris, pH 7.5, 0.1 % BSA), three times in TBS (plus 0.1 % Tween-20), rinsed in PBST and blocked in 5% goat serum in PBS, 1% BSA for 30 min at room temperature. Slides were washed in PBS, incubated in primary antibody (mouse anti α-sm-actin, 1:100, DAKO) for 1h at room temperature, rinsed in PBST followed by application of secondary antibody, Alexa Fluor 488 (Molecular Probes, UK) diluted 1:1000 in PBS for 30min. Slides were washed in
PBST, once in deionised water, and mounted in Vectashield Mounting Medium containing DAPI (Vector Laboratories, USA).

QTELI-FISH signals were quantified using computer controlled fluorescence acquisition. Sampling areas within the media were used to determine integration times, and background and threshold levels were equalised between slides. Gain and exposure times were established within a linear range of signal intensity. Typically, integration times of 500–2000 ms for Cy3 signals, 2–5 ms for DAPI nuclear staining and 100-500 ms for Alexa Fluor conjugated anti-smooth muscle actin were used. For each slide all Cy3 signals utilized the same integration and exposure times. For normal tissue sections both medial and intimal nuclei were chosen randomly as long as the Cy3 signals were in sharp focus. In diseased sections, the nuclei within the media were chosen randomly, but within the thickened intima nuclei with the brightest signals were selected. This was necessary as most nuclei in the diseased intima contained few and faint Cy3 signals hardly observable over the background.

For each nuclear image, fluorescent signals were measured using ImageJ (http://rsb.info.nih.gov/ij/). Briefly, for each telomeric signal within the area of the nucleus (as determined by DAPI staining) the mean Cy3 signal and the area of the Cy3 spot was determined. Similarly background fluorescence was determined for an area of the nucleus containing no noticeable Cy3 signal. Following background fluorescence subtraction, the sum of the mean fluorescence and the area of the Cy3 signal represented the total fluorescence for each given telomere. The individual telomere signals for the whole nucleus were summated and then divided by the total
area of the nucleus. This corrected for differences in the cutting plane of each nucleus and so different nuclei on the same slide could be directly compared.

**Senescence associated β-Galactosidase staining**

Cells were washed twice in PBS, fixed for 10 minutes at 37°C in 4% paraformaldehyde, washed twice in PBS and incubated for 24h at 37°C in SAβG staining solution (1mg/ml X-Gal, 5mmol/l potassium ferrocyanide, 5mmol/l potassium ferricyanide, 150mmol/l NaCl, 2mmol/l MgCl₂, 40mmol/l citrate (titrated to pH 6.0 with NaH₂PO₄)). Slides were washed in PBS, water and mounted in DAPI fluorescence mounting medium (DAKO). Tissue specimens were washed twice in PBS, incubated for 24h at 37°C in SAβG staining solution, washed twice in PBS and fixed in 10% formalin for 24h.

**Retrovirus-based expression of hTERT in human VSMCs**

Human TERT cDNA was cloned into the retroviral vector pBMN IRES puro² and transfected into Bosc23 ecotropic packaging cells using Superfect (Qiagen). Virus was recovered 48 hours later and used to infect GPenv Am12 amphotropic packaging cells for 3h in medium containing 8µg/ml polybrene. Virus was harvested at 48 hours and used to infect human VSMCs for 16 hours in medium containing 8µg/ml polybrene. VSMCs were selected in medium containing 0.5µg/ml puromycin, resistant cells pooled, and cultures maintained in puromycin-containing media thereafter.
RTQ-TRAP assay for telomerase activity

Telomerase activity was measured by RTQ-TRAP assay \(^3, 4\) using TS (5’-AATCCGTGCAGCAGAGTT-3’) and ACX (5’-GCGCGG(CTTACC)\(\_\)CTAACC-3’) primers\(^3\). Cell pellets were washed in ice-cold PBS, resuspended in lysis buffer (TRAPeze 1x CHAPS lysis buffer, Chemicon Int., Temecula, CA) containing 1U/\(\mu\)l of ribonuclease inhibitor (RNasin, Promega Corp., Madison WI) and incubated for 30 min at 4\(^\circ\)C. Lysates were centrifuged at 12,000g for 20min at 4\(^\circ\)C and supernatants collected. Protein content of supernatants was measured using the De Protein Assay (BioRad). Total protein per reaction (2-200ng) was in the linear range of the assay. Extension of TS primers was performed at 30\(^\circ\)C for 30 min. PCR amplification was performed as previously described \(^4\) using the HotStarTaq DNA polymerase (0.62U/reaction, Qiagen GmBH, Hilden, Germany) and a Rotor Gene 3000 real time thermocycler (Corbett Research, Australia). Negative controls were generated by heating cell lysates at 80\(^\circ\)C for 10 min prior to extension. Dilutions of Jurkat cell lysates were used to generate standard curves. Telomerase activity in VSMCs lysates was extrapolated from the standard curve. Telomerase activity from control and BHP-treated VSMCs was expressed as percent of values in untreated samples. Negative controls were generated by heating cell lysates at 80\(^\circ\)C for 10 min prior to extension, in order to inactivate telomerase. Residual signal in heated lysates was less than 0.1% of telomerase activity generated signal.

Flow cytometric analysis for determination of ROS and mitochondrial mass

ROS content in normal VSMCs (passage 2-5), SAβG-positive replicatively senescent cells (passage 12-16), plaque VSMCs (passage 2-4) or t-BHP treated VSMCs (40 \(\mu\)M, 1 h), was measured in triplicates using the probe 2’7’-dichlorofluorescein
diacetate (H$_2$-DCFDA; Molecular Probes). The cells were incubated for 1 h with 2 
µM H$_2$-DCFDA, rinsed in PBS and trypsinized for flow cytometric analysis. 
Alternatively, cells were rinsed in PBS and photographed under a fluorescence 
microscope. The fluorescent dye 10-$n$-Nonyl-Acridine Orange (NAO; Molecular 
Probes) was used to measure mitochondrial mass as previously described$^5$. 
Autofluorescence values from each cell group (no fluorophore control) were 
subtracted from total fluorescence.
References


Legends to Online Figures

Online Figure 1

Identification of plaque-derived cells as VSMCs by immunocytochemical staining.

Plaque derived VSMCs were incubated with antibodies to $\alpha$-smooth muscle actin (A), calponin (B), smooth muscle myosin (C), vimentin (D), desmin (E), von Willebrand factor (F), irrelevant antibody (G) and no primary antibody (H), prior to labelling with FITC-conjugated secondary antibody. DAPI was used to label nuclei.

Online Figure 2

$t$-BHP and replicative arrest induces oxidant stress in VSMCs

ROS levels (A) or mitochondrial mass (B) in low passage normal VSMCs (CTR), plaque VSMCs (PLQ) and normal VSMCs at replicative senescence (SEN). Fluorescence levels were analysed by flow cytometry using the redox sensitive dye H$_2$-DCFDA (ROS) or 10-$n$-nonyl-Acridine Orange (mitochondrial mass). Fluorescence values are expressed as arbitrary units (AU) and results are means±SEM of 4 independent experiments. C) ROS levels in normal VSMCs either untreated (CTR) or treated with 40 $\mu$M BHP analysed by fluorescence microscopy (left panels) or quantified by flow cytometry (right panel). Fluorescence values are expressed as arbitrary units (AU). Results are means ± SEM of at least 4 independent experiments. *p<0.05 vs control (CTR), **p<0.01 vs control (CTR).
Online Figure 1
Online Figure 2