Telomerase Activation in Atherosclerosis and Induction of Telomerase Reverse Transcriptase Expression by Inflammatory Stimuli in Macrophages

Florence Gizard, Elizabeth B. Heywood, Hannes M. Findeisen, Yue Zhao, Karrie L. Jones, Céline Cudejko, Ginell R. Post, Bart Staels, Dennis Bruemmer

Objective—Telomerase serves as a critical regulator of tissue renewal. Although telomerase activity is inducible in response to various environmental cues, it remains unknown whether telomerase is activated during the inflammatory remodeling underlying atherosclerosis formation. To address this question, we investigated in the present study the regulation of telomerase in macrophages and during atherosclerosis development in low-density lipoprotein receptor-deficient mice.

Methods and Results—We demonstrate that inflammatory stimuli activate telomerase in macrophages by inducing the expression of the catalytic subunit telomerase reverse transcriptase (TERT). Reporter and chromatin immunoprecipitation assays identified a previously unrecognized nuclear factor-κB (NF-κB) response element in the TERT promoter, to which NF-κB is recruited during inflammation. Inhibition of NF-κB signaling completely abolished the induction of TERT expression, characterizing TERT as a bona fide NF-κB target gene. Furthermore, functional experiments revealed that TERT deficiency results in a senescent cell phenotype. Finally, we demonstrate high levels of TERT expression in macrophages of human atherosclerotic lesions and establish that telomerase is activated during atherosclerosis development in low-density lipoprotein receptor-deficient mice.

Conclusion—These results characterize TERT as a previously unrecognized NF-κB target gene in macrophages and demonstrate that telomerase is activated during atherosclerosis. This induction of TERT expression prevents macrophage senescence and may have important implications for the development of atherosclerosis.

Key Words: atherosclerosis  ■  immune system  ■  macrophages  ■  telomerase

Telomeres are repetitive DNA sequences that cap linear chromosomes to prevent degradation, end-to-end fusion, and rearrangement.1,2 Owing to the biochemistry of incomplete DNA replication, each cycle of DNA replication leaves 50 to 200 bp at each 3’ end unreplicated. The solution to this end-replication problem adopted by most organisms is the use of telomerase, a telomere-specific DNA polymerase that catalyzes the addition of TTAGGG repeats onto telomeric DNA through the activity of the telomerase reverse transcriptase (TERT).3 However, the majority of human adult somatic cells are thought to transcriptionally repress TERT, resulting in telomere shortening and cellular senescence.4 In contrast, stem cells and most cancer cell lines constitutively overexpress TERT, manifesting high levels of telomerase activity, which confers an apparently indefinite lifespan.5 Therefore, through its action in synthesizing telomeres at the end of the chromosome, constitutive telomerase activity is required to maintain the replicative capacity of progenitor cells during tissue renewal.6,7

Although most adult cells display low basal telomerase activity, a growing body of evidence indicates that the catalytic subunit TERT is tightly regulated in its expression and inducible in various cell types in response to changes in environmental cues.8 For example, in mature CD4+ and CD8+ T cells, telomerase activity is highly induced following antigenic stimulation.9,10 Similarly, it has recently been reported that telomerase is reactivated on maturation of dendritic cells.11 These observations indicate that telomerase activation may occur at critical stages of lymphoid and myeloid cell activation, and it has been postulated that this inducible telomerase activation may augment their function during immune responses.12-14

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Intriguingly, telomerase activity has recently been demonstrated to be increased in neutrophils isolated from coronary atherosclerotic plaques of patients with unstable angina. At the cellular level, TERT is expressed in all cell types participating in the atherosclerotic disease process, including macrophages. However, whether telomerase is activated during atherosclerosis development and the putative molecular mechanisms underlying this activation remain unknown. In the current study, we sought to define the regulation and function of TERT in macrophages and investigate whether telomerase is activated during the inflammatory remodeling underlying atherosclerosis development.

Materials and Methods

Reagents, Plasmids, and Adenoviral Constructs

Reagents were obtained from the following suppliers: lipopolysaccharide (LPS) from Sigma-Aldrich; oxidized and nonoxidized low-density lipoprotein (oxLDL) from Intracel; and tumor necrosis factor-α (TNF-α), interleukin (IL)-6, and IL-1β from Biologic or R&D Systems. The cytomegalovirus (CMV)-driven dominant-negative (DN) inhibitor kappa B-alpha (IκBα) expression vector (CMV-DN-IκBα) overexpressing a nonphosphorylatable N-terminal deletion mutant of IκBα was kindly provided by Dr M. Chaisson. The adenovirus overexpressing a dominant-negative IκBα mutant (Ad-DN-IκBα) was obtained from Vector Bioslabs. The adenovirus overexpressing green fluorescent protein (Ad-GFP) and the TERT reporter constructs were used as described. The luciferase reporter construct driven by multiple copies of the NF-κB consensus sequence (8×NF-κB-Luc) was obtained from Clontech.

Cell Culture

The human U937 monocyte and murine RAW 264.7 macrophage cell lines were obtained from American Type Culture Collection and cultured in Dulbecco’s modified Eagle’s medium (DMEM) or RPMI-1640 medium supplemented with 4 mmol/L glutamine, 10% fetal bovine serum, and antibiotics, respectively. Differentiation of U937 monocytes into macrophages was induced by incubating monocytes for 16 hours with 0.1 mmol/L phorbol-12-myristate-13-acetate (Sigma-Aldrich) followed by 24 hours of incubation in complete medium before treatment with inflammatory stimuli. Mouse peritoneal macrophages (MPM) were elicited as described and incubated for 24 hours in DMEM supplemented with 10% lipoprotein–deficient serum (LPDS; Intracel). For adenoviral infections, 1×10⁵ MPM were seeded in 6-well plates, and viral stocks of Ad-GFP or Ad-DN-IκBα (25 plaque-forming units [PFU]) were immediately added to the cells. Four hours after infection, MPM were washed in PBS, reincubated in DMEM containing 1% LPDS overnight, and stimulated as indicated. All experiments were repeated at least 3 times in duplicate or triplicate using different cell preparations.

Analysis of Telomerase Activity

Telomerase activity was analyzed using a quantitative Telomerase Detection Kit (Allied Biotech, Inc.) according to the manufacturer’s instructions. Polymerase chain reactions (PCRs) were performed using whole-cell extract containing 0.4 μg of protein and 36 cycles of denaturation/annealing/extension steps.

Reverse Transcription and Quantitative PCR

Transcript levels of target genes were quantified by real-time reverse transcription–PCR as described. All primer sequences are provided in Supplemental Table I, available online at http://atvb.ahajournals.org.

Western Blotting

Western blotting was performed using antibodies for TERT (600-401-252, Rockland) or GAPDH (sc-25778, Santa Cruz Biotechnol- ogy) as described. The antibody detecting human TERT (600-401-252, Rockland) has previously been validated to specifically detect endogenous TERT expression.

Transient Transfection

RAW 264.7 cells were transfected using Lipofectamine 2000 (Invitrogen) as described. Following transfection, cells were cultured in Opti-MEM supplemented with 10% LPDS overnight before stimulation with oxLDL or LPS. Luciferase activities were analyzed using the Dual-Luciferase Reporter Assay system (Promega).

Chromatin Immunoprecipitation Assays

Chromatin immunoprecipitation assays were performed as described using the following antibodies: anti-p65 (sc-8008 or sc-372), anti-p50 (sc-8414), and nonimmune mouse or rabbit IgG (sc-202 or sc-202, respectively), all from Santa Cruz Biotechnology. Extracted DNA was PCR amplified using primer pairs covering the indicated promoter elements (Supplemental Table I). Primer pairs surrounding the NF-κB element in the monocyte chemotactic protein-1 (MCP-1) promoter or a distal region in the β-actin and TERT promoters served as positive and negative controls, respectively.

Immunohistochemistry

Segments of normal human coronary arteries or arteries containing atherosclerotic lesions were harvested from hearts during autopsy. Tissues were fixed in 4% phosphate-buffered formaldehyde and embedded in paraffin. Transverse sections (8 μm) were collected, and immunohistochemical analysis was performed using a TERT antibody (582005; Calbiochem) followed by counterstaining with hematoxylin as described. For confocal microscopy, sections were incubated with antibodies against TERT and CD68 (M0876, Dako Cytomation). Sections were subsequently incubated with Alexa Fluor 488–conjugated goat anti-rabbit IgG (A11008, Invitrogen) and Alexa Fluor 594–conjugated goat anti-mouse IgG (A11005, Invitrogen). All studies on human tissues were performed with the approval of the University of Kentucky Institutional Review Board.

Analysis of Cellular Senescence

TERT−/− mice were obtained from the Jackson Laboratory (stock number 005423). MPM from first-generation TERT−/− and littermate wild-type mice were elicited as described. Cells from individual mice were pooled, plated on 6-well cell culture dishes, and maintained in DMEM supplemented with 10% fetal bovine serum. Senescence-associated β-galactosidase activity was analyzed using the Senescence Cells Histochemical Staining Kit (CS0030, Sigma-Aldrich). Representative pictures were acquired, and the signal intensity was quantified using image analysis software. The signal intensity level of cyan staining was normalized to background level and expressed as the mean signal intensity±SD. Experiments were repeated at least 3 times using different macrophage preparations. All studies on animals were performed with the approval of the University of Kentucky Institutional Animal Care and Use Committee.

Analysis of Telomerase Activity In Vivo

Male low-density lipoprotein receptor–deficient (LDLr−/−) mice were obtained from the Jackson Laboratory (stock number 002207). At 12 weeks of age, mice (n=8/group) were randomized to receive either standard laboratory chow diet or a diet supplemented with saturated fat and cholesterol (TD88137, Harlan Teklad). After 12 weeks of diet feeding, mice were euthanized, and aortic tissues were analyzed for telomerase activity using a PCR-based telomeric repeat amplification protocol (TelotAGGG Telomerase PCR ELISA, Roche Applied Biosystems) as described.

Statistics

Statistical differences between different groups were assessed by 1-way ANOVA followed by Tukey test assays or the Student t test. Probability values <0.05 were considered to be statistically significant. Results are expressed as mean±SD or SEM as indicated.
**Results**

**Proinflammatory Stimuli Induce TERT Expression and Telomerase Activity in Macrophages**

On the basis of the evidence that TERT confers the catalytic activity of telomerase and our previous observation that other components of the telomerase complex are constitutively expressed but not regulated in vascular cells, we first investigated the regulation of TERT in macrophages. In these experiments, stimulation of differentiated human U937 macrophages with the proinflammatory mediators LPS, oxLDL, TNF-α, or IL-1β significantly increased TERT mRNA expression (Figure 1A) and telomerase activity (Figure 1B). The induction of TERT transcript levels by LPS, oxLDL, and TNF-α paralleled inducible telomerase activity. In contrast, IL-1β only modestly increased TERT mRNA, albeit a potent induction of telomerase activity, pointing to a potentially distinct posttranscriptional induction of telomerase activity by IL-1β. More detailed time-course experiments revealed that the induction of TERT expression and activity by oxLDL was transient (maximal levels after 3 hours) (Supplemental Figure I) and dose dependent (Figure 1C to 1E). Furthermore, a comparable induction of TERT mRNA expression and telomerase activity by proinflammatory mediators was observed in primary MPM and human macrophages differentiated in vitro from monocytes of healthy donors (Supplemental Figures II and III). In concert, these studies establish a solid induction of TERT expression and telomerase activity in response to inflammatory stimulation of macrophages.

**NF-κB Signaling Mediates the Induction of TERT Expression in Response to oxLDL and LPS**

To investigate the transcriptional mechanisms involved in the induction of TERT expression in macrophages, we analyzed 5′-deletion series of a luciferase reporter driven by a 2-kb TERT
promoter fragment. As depicted in Figure 2A, treatment of macrophages with oxLDL significantly induced the activity of the -2.0-kb but not the 18-bp TERT promoter. oxLDL-dependent TERT promoter activation was maintained on 5' deletion to -776 bp, indicating that a 202-bp promoter region between -776 and -574 conferred the induction of the TERT promoter activity by oxLDL.

Sequence analysis of this TERT promoter region identified a highly conserved NF-κB response element at -592/-580. Moreover, transient transfection of an NF-κB-driven reporter construct (8×NF-κB-Luc) confirmed a potent increase in NF-κB transcriptional activity in response to oxLDL (Figure 2A, lower panel). To more definitively address the role of NF-κB signaling for the activation of the TERT promoter in macrophages, we next performed transactivation assays using a TERT promoter construct (-776TERTLuc, +18TERTLuc, or 8×NF-κB-Luc reporter construct and either an empty CMV vector or a CMV-DN-IκBα expression vector. Following transfection, macrophages were cultured in 10% LPDS and treated with oxLDL (10 μg/mL) (B) or LPS (100 ng/mL) (C). Following stimulation, cells were harvested and analyzed for luciferase activities as in A. Data are expressed as normalized luciferase activities and presented as mean±SD percentage increase over basal TERT promoter activity (P<0.05 versus untreated cells [A] or CMV empty vector [B and C]; ns indicates not significant). D, Peritoneal macrophages were infected with 25 plaque-forming units (PFU) adenovirus overexpressing green fluorescent protein (Ad-GFP) Ad-GFP or Ad-DN-IκBα and treated with LPS (100 ng/mL) for 24 hours. TERT mRNA expression was analyzed and normalized to transcription factor IIb (TFIIB) transcript levels. Data are presented as mean±SD percentage increase compared with untreated Ad-GFP-infected cells (n=3, *P<0.05 versus vehicle).
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**dently performed experiments.** or on chromatin immunoprecipitated with nonimmune

**include** PCR performed on nonprecipitated genomic DNA

**the recruitment of both NF- 

**region encompassing an NF- 

**that oxLDL induces a strong recruitment of p65 and p50 to a 

**tin immunoprecipitation analysis in primary MPM revealed 

**in the human TERT promoter (Figure 3). Similarly, chroma-

**proximal NF- 

**/H11002 580 in 

**TERT-deficient mice significantly upregulated senescence-

**replicative senescence.** Furthermore, TERT-deficient macro-

**replicative senescence.** Including p16, p21, and retinoblastoma protein (RB) transcript levels in 

**wild-type and TERT-/- macrophages. Target gene mRNA 

**expression was analyzed by real-time reverse transcription–PCR 

**and normalized to transcript levels of the housekeeping gene transcription factor IB (TFIIB). Data are presented as mean±SEM percentage increase compared with wild-type macro-

**phages (n=3,  P<0.05).**

**NF-kB subunits are recruited to the consensus site 

**NF-kB site at 

**p21, and the retinoblastoma protein (Figure 4C). Collectively, 

**induces cellular senescence. A and B, MPM from TERT 

**/H11002 -galactosidase activity in TERT 

**performed on nonprecipitated genomic DNA (input) or on chromatin immunoprecipitated with nonimmune IgG. The agarose gels shown are representative of 3 independently performed experiments.

**Figure 3. Inflammatory stimuli induce NF-kB recruitment to the 

**TERT promoter in macrophages. Differentiated U937 macro-

**phages were treated for 2.5 hours with LPS (100 ng/mL), oxLDL 

**(50 μg/mL), or TNF-α (50 ng/mL). Chromatin immunoprecipita-

**tions were performed with antibodies raised against p65 or p50, 

**followed by PCR amplification using primer pairs that cover the 

**proximal NF-kB site in the human TERT promoter. Controls 

**included PCR performed on nonprecipitated genomic DNA

**NF-kB Is Recruited to the TERT Promoter 

**in Macrophages

**We next performed chromatin immunoprecipitation assays 

**using primer pairs that cover the NF-kB site at -592/-580 in 

**the human TERT promoter to determine whether NF-kB subunits 

**are recruited to the endogenous TERT promoter. These assays 

**confirmed that LPS, oxLDL, and TNF-α induce the recruitment of both NF-kB subunits to the consensus site 

**in the human TERT promoter (Figure 3). Similarly, chroma-

**tin immunoprecipitation analysis in primary MPM revealed 

**that oxLDL induces a strong recruitment of p65 and p50 to a 

**region encompassing an NF-kB site at -250/-238 in the 

**murine TERT promoter (Supplemental Figure IV). Taken 

**together, these experiments demonstrate that inflammatory stimuli 

**induce NF-kB activation, leading to the subsequent transcription of the TERT gene.

**Genetic TERT Deficiency Induces 

**Macrophage Senescence

**To investigate the functional role of inducible TERT expression in the macrophage, we next analyzed phenotypes in cells isolated from mice genetically deficient for the TERT locus. As 

**depicted in Figure 4A, MPM isolated from first-generation 

**TERT-deficient mice significantly upregulated senescence-

**associated β-galactosidase activity, an established biomarker of 

**replicative senescence.**

**TERT Is Expressed in Macrophages of Human 

**Coronary Artery Atherosclerotic Lesions

**To corroborate that TERT is expressed in complex athero-

**sclerotic lesions in vivo, we performed immunohistochemical analysis on human coronary arteries. Consistent with earlier reports, TERT expression was negligible in normal human coronary arteries (Figure 5A) but highly expressed in athero-

**sclerotic lesions (Figure 5B). Particularly high levels of 

**TERT expression were observed in the macrophage-rich 

**shoulder region of advanced atherosclerotic lesions (Figure 

**5B). Using immunofluorescent analysis, Figure 5C depicts 

**representative colocalization of TERT with macrophage 

**CD68 immunoreactivity in the atherosclerotic lesion. These 

**observations confirm that TERT is expressed in macrophages 

**of human atherosclerotic lesions.

**Telomerase Activity Is Increased During 

**Atherosclerosis Formation

**The increased expression of TERT in macrophages of athero-

**sclerotic lesions raised the question of whether TERT
expression in atherosclerosis is functional. To address this question, we analyzed TERT activity in aortic tissues isolated from LDLr−/− mice fed a control diet or an atherogenic Western diet for 12 weeks. Quantification of vascular telomerase activity (Figure 6, left panel) revealed that the development of atherosclerosis was associated with a more than 13-fold increase in telomerase activity. A typical Southern blot from 4 representative samples is shown in Figure 6, right panel, and confirms increased vascular telomerase activity in LDLr−/− mice fed an atherogenic diet. In concert, these observations demonstrate that telomerase is activated during atherosclerosis development, a process driven primarily by macrophages.

Discussion

Although it is well established that somatic cells repress TERT, resulting in telomere attrition,4 accumulating evidence suggests that many cell types are capable of reactivating telomerase in response to environmental cues.9–11 Although telomerase activation has been described during adaptive immune responses and enhances immune function of lymphoid cells,12–14 little is known about the regulation and function of telomerase during macrophage inflammation. In the current study, we provide initial evidence that inflammatory mediators induce TERT expression and telomerase activation during macrophage inflammation. TERT is expressed in macrophages of human atherosclerotic lesions, and
we further establish that telomerase is activated during experimental atherosclerosis formation. Moreover, functional experiments demonstrate that genetic TERT deficiency in macrophages induces a phenotype of cellular senescence, pointing to a potentially important role of inducible TERT expression during inflammation.

TERT confers the catalytic activity of telomerase and is primarily regulated through transcriptional mechanisms. Consistent with this evidence was our observation that the induction of TERT transcript levels by oxLDL, LPS, or TNF-α in macrophages paralleled a concomitant telomerase activation. Considering these findings, we sought to define the transcriptional mechanisms underlying inducible TERT expression in the macrophage. Using 5’-deletion analysis, we identified a proximal promoter region that conferred TERT transcription. Sequence analysis of this region revealed a highly conserved NF-κB response element, and inhibition of NF-κB signaling completely abolished TERT expression during macrophage inflammation. Moreover, NF-κB was rapidly recruited to this site in response to inflammatory stimuli, characterizing TERT as a previously unrecognized bona fide NF-κB target gene in macrophages. Although these findings establish that NF-κB signaling induces TERT transcription in macrophages, we cannot exclude the possibility that stimulus-dependent posttranscriptional/posttranslational mechanisms might contribute to telomerase activation in this cell type. Interestingly, IL-1β only modestly increased TERT transcript levels but potently induced telomerase activity, pointing to possible distinct mechanisms governing IL-1β-induced telomerase activation in macrophages. Therefore, potential posttranscriptional/posttranslational mechanisms of telomerase activation by IL-1β in macrophages remain to be further investigated, but they may involve Akt-dependent phosphorylation and nuclear import of TERT, which have both been implicated in telomerase activation in certain cell types.

An intriguing question that arises from the observation that TERT is expressed during macrophage inflammation relates to the regulation of telomerase activity during inflammatory remodeling underlying atherosclerosis formation. Our observations presented herein indicate that macrophages in the shoulder region of advanced human atherosclerotic plaques express high levels of TERT. These findings are consistent with recent data published by Matthews et al., who noted that TERT expression is readily detectable in lesional macrophages. In contrast, other cell types present in the human advanced atherosclerotic lesion, including endothelial cells and smooth muscle cells of the fibrous cap, are thought to express low levels of TERT. Using LDLr−/− mice, our data extend these observations and identify a more than 13-fold increase in telomerase activity during lesion formation. Similarly, Narducci et al recently documented high telomerase activity in neutrophils isolated from coronary artery plaques of patients with unstable angina. In concert with our observations in LDLr−/− mice, these studies provide compelling evidence for the activation of telomerase during atherosclerosis development and support the notion that this telomerase activation originates primarily from macrophages.

Limiting TERT expression results in cellular senescence, a state that is accompanied by changes in cell morphology, function, and gene expression programs. In consonance with this notion, we observed that TERT deficiency in macrophages induces senescence-associated β-galactosidase activity and gene expression of p16, p21, and the retinoblastoma protein, both established biomarkers of cellular senescence. This finding indicates that transient TERT expression during inflammation may prevent senescence of the macrophage, potentially to maintain cellular responsiveness during immunity. Indeed, senescent macrophages display altered immune functions, and overexpression of TERT in fibroblasts has recently been demonstrated to increase IL-1β, IL-6, and IL-8 expression, indicating that TERT may not only prevent senescence but also participate in a feed-forward loop for inflammatory gene expression. Considering that TERT extends lifespan without increasing telomere length, whereas TERT disruption induces senescence without telomere shortening, it has been questioned whether the activity of TERT to prevent senescence is dependent on its function to elongate telomeres. Alternatively, there is an increasing appreciation of telomere-independent activities of TERT, and recent experiments have demonstrated that overexpression of TERT activates gene expression programs and induces chromatin remodeling. Although the molecular mechanisms underlying these noncanonical functions remain unclear, the broader changes in gene expression affected by TERT point to a regulation of signaling pathways or transcriptional mechanisms. With respect to inflammatory signaling, this concept is further supported by the finding that TERT physically interacts with NF-κB, which may provide a reasonable mechanism underlying the activation of inflammatory genes by TERT.

The expression and activation of TERT in macrophages presented in this study provide important justification for further investigating the physiological function of TERT in inflammation. Moreover, the activation of telomerase in atherosclerosis described here and the demonstration that telomere attrition prevents atherosclerosis in apolipoprotein E–deficient mice, combined with the reported telomere shortening in circulating leukocytes of patients with coronary artery disease, indicate...
that the association of TERT expression, telomerase activation, and telomere length is more complex than previously anticipated. Continued investigation of the role of TERT in vascular biology will likely provide new insights into how TERT functions during atherosclerosis development.

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Disclosures
None.

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**Supplemental Table I:** Primer sequences used in this study.

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Supplemental Figure I. Time-dependent induction of TERT expression and telomerase activity by oxidized LDL in human macrophages. Differentiated human U937 macrophages were treated with LPS (100 ng/ml) or oxidized LDL (50 µg/ml). At the indicated time points, TERT mRNA expression levels (upper panel) and telomerase activity (lower panel) were analyzed. TERT mRNA expression was quantified by real-time RT-PCR and normalized to transcript levels of the house-keeping gene TBP. Whole cell proteins were analyzed for TERT activity by real-time PCR as described in “Materials and Methods”. All data are presented as mean ± SEM percent increase compared to untreated cells (n=3, * P<0.05).
Supplemental Figure II. Induction of TERT expression and telomerase activity by inflammatory stimuli in primary murine macrophages. Mouse peritoneal macrophages were treated with oxidized LDL (oxLDL, 50 µg/ml) or LPS (100 ng/ml). TERT mRNA expression levels (upper panel) were analyzed by real-time RT-PCR and normalized to transcript levels of 28S rRNA. Telomerase activity (lower panel) was quantified by real-time PCR as described in “Materials and Methods”. All data are presented as mean ± SEM percent increase compared to untreated cells (Control), (n=3, * P<0.05).
Supplemental Figure III. Induction of TERT expression by inflammatory stimuli in primary human macrophages. Mononuclear cells were isolated from blood of healthy normolipidemic donors. After Ficoll gradient centrifugation, monocytes were suspended in RPMI 1640 medium containing gentamycin (40 µg/ml), glutamine (0.05%) (Sigma), and 5% pooled human serum. Cells were cultured at a density of $3 \times 10^6$ cells/well in 6-well culture dishes. Differentiation of monocytes into macrophages occurred spontaneously by adhesion of cells and culture for 12 days. Mature macrophages were cultured in 0.5 % serum overnight and treated with LPS (100 ng/ml) or TNFα (5 ng/ml). TERT mRNA expression was quantified by real-time RT-PCR and normalized to transcript levels of 28S rRNA. Data are presented as mean ± SEM percent increase compared to untreated cells (Control), (n=3, * P <0.05 vs. Control).
**Supplemental Figure IV.** Oxidized LDL induces NF-κB binding to the endogenous TERT promoter in primary murine macrophages. Mouse peritoneal macrophages were treated for 2.5 h with oxidized LDL (50 µg/ml). Chromatin immunoprecipitations were performed with antibodies raised against p50 or p65, followed by PCR amplification using primer pairs that cover the proximal NF-κB site in the murine TERT promoter (mTERT-NF-κB element). Controls included non-precipitated genomic DNA (Input) or immunoprecipitations performed with non-immune IgG (IgG). Additional controls for specificity included PCR amplification with primer pairs encompassing the NF-κB site in the murine MCP-1 promoter (mMCP-1-NF-κB element, positive control), a distal unrelated element in the murine TERT promoter (mTERT-distal element, negative control), or the unrelated murine β-actin promoter (mβ-actin element, negative control). All ethidium bromide–stained agarose gels shown are representative of three independently performed experiments.