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Short telomeres protect from diet-induced atherosclerosis in apolipoprotein E-null mice

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ABSTRACT

By imposing a replicative defect in most somatic cells, gradual telomere attrition during aging is thought to progressively impair cellular function and viability and may contribute to age-related disease. Immune cells play important roles in all phases of atherosclerosis, a multifactorial disease that prevails within the elderly. Because shorter telomeres have been found in circulating blood leukocytes of human patients with advanced coronary atherosclerosis, it has been suggested that telomere shortening may predispose the organism to atheroma development. In this study, we assessed the impact of telomere attrition on atherogenesis induced by dietary cholesterol in apolipoprotein E (apoE)-deficient mice, a well-established model of experimental atherosclerosis that recapitulates important aspects of the human disease. Our study shows that late-generation mice doubly deficient in apoE and telomerase RNA experience telomere attrition and a substantial reduction of atherosclerosis compared with control mice with intact telomerase, in spite of sustained hypercholesterolemia in response to the atherogenic diet. Short telomeres impaired the proliferation of both lymphocytes and macrophages, an important step in atherosclerosis development. Therefore, telomere exhaustion resulting in replicative immunosenescence may serve as a mechanism for restricting atheroma progression.

Key words: apoE • smooth muscle cells • SMα-actin

Shortening of telomeres, the ends of eukaryotic chromosomes, limits the proliferative potential of primary cells in vitro (1–3). Telomere attrition in humans has been documented in various tissues during aging and in some chronic diseases associated with high cell turnover (1–3). Aging is a major risk factor for atherosclerosis and related diseases (e.g., myocardial infarction, heart failure, and stroke; refs 4, 5). Both genetic and environmental factors, i.e., elevated plasma cholesterol level, hypertension, and diabetes, contribute to atherosclerosis. The development of atherosclerotic lesions is initiated by transendothelial migration and activation of circulating monocytes and lymphocytes at the sites of vessel injury

(4, 5). Recruited leukocytes release inflammatory chemokines and cytokines that promote smooth muscle cell (SMC) proliferation and migration toward the atherosclerotic lesion, thus further contributing to atheroma growth (4–7). It has become increasingly evident that both adaptative and innate immune mechanisms modulate the inflammatory response during atherosclerosis (8, 9).

It has been well established that telomere attrition imposes replicative senescence to immune cells (10). Likewise, several in vitro studies have shown that limited proliferative capacity of passaged human endothelial cells and SMCs correlates with progressive telomere shortening, and forced alteration of telomerase activity affects the lifespan and replicative potential of these cells (11–15). Notably, induction of telomerase activity by chronic hypoxia may extend the lifespan of human SMCs (16), and constitutive human telomerase reverse transcriptase expression enhances the regenerative capacity of endothelial progenitor cells (17). Human and animal studies implicating telomere dysfunction in the pathogenesis of cardiovascular disease include the following: *1*) telomerase is activated selectively in aortic tissue of genetically hypertensive rats before the onset of hypertension (15); *2*) mice with critically short telomeres develop cardiac dilation and heart failure (18), and failing human hearts have telomeres that are 25% shorter than age-matched controls (19); *3*) age-dependent telomere shortening has been observed in human vascular endothelium (20), and telomere exhaustion is greater in atherosclerotic lesions of human arteries subjected to higher atherogenic hemodynamic stress (11); *4*) telomere length in human aorta negatively correlates with atherosclerosis grade, although this relationship is not significant after adjustment for age (21); and *5*) leukocytes of patients with advanced coronary atherosclerosis have shorter telomeres than age-matched subjects with normal coronary angiograms (22), and this is associated with increased risk of heart disease and higher mortality rate (23, 24). Although these findings raise the possibility that telomere attrition may be a primary abnormality that predisposes the organism to atherosclerosis, no studies are available showing such causal link. The goal of the present study was to test the impact of telomere exhaustion on atherogenesis by using genetically modified mice.

MATERIALS AND METHODS

Mice

Care of animals was in accordance with institutional guidelines. Mice singly deficient for apoE (25, 26) (C57BL/6J background, Taconic M&B) and the RNA component of telomerase (Terc) (27) (mixed C57BL/6J x 129Sv background) were mated, and the double heterozygous F1 offspring were intercrossed. F2 mice were genotyped by PCR analysis, and brother-sister mating of $Terc^{-/-}apoE^{-/-}$ and $Terc^{+/+}apoE^{-/-}$ mice was performed to obtain up to fourth generation $Terc^{-/-}apoE^{-/-}$ and $Terc^{+/+}apoE^{-/-}$ mice. After weaning, the mice were maintained on a low-fat standard diet (2.8% fat, Panlab, Barcelona, Spain). At 2 months of age, mice received for 1.5 months an atherogenic diet containing 15.8% fat, 1.25% cholesterol, and 0.5% sodium cholate (S4892-S010, Ssniff, Soest, Germany). Blood was withdrawn before and after the high-fat diet to measure plasma cholesterol levels using enzymatic procedures (Sigma, St. Louis, MO). In agreement with previous studies showing reduced body size and lifespan of late-generation $Terc^{-/-}$ mice (28), third generation $Terc^{-/-}apoE^{-/-}$ mice displayed reduced body size when compared with $Terc^{+/+}apoE^{-/-}$ counterparts [14- to 16-month-old maintained on control chow, body weight (g) = 19.04±0.76, n=7, and 29.04±1.16, n=7, respectively, P<0.00001]. Moreover,

50% of fourth generation $Terc^{-/-}apoE^{-/-}$ mice that were initially included in our study died during the 6 wk period of fat feeding, whereas all fat-fed $Terc^{+/+}apoE^{-/-}$ counterparts survived until completion of the study.

Quantification of atherosclerosis and immunohistochemistry

Fat-fed mice were euthanized, and aortic atherosclerosis was quantified by Oil Red O staining and histological examination essentially as described previously (29). In one set of animals, vessels were fixed in situ with 4% paraformaldehyde, then they were removed and fixed overnight, and finally were stained with Oil Red O solution (0.2% in 80% MetOH) (Sigma). The outer adventitia was carefully removed, and then specimens were opened longitudinally and pinned onto a flat surface and photographed with a Sony DKC-CM30 camera (Tokyo, Japan) mounted on a Zeiss Stemmi 2000-C dissecting scope (Jena, Germany). To determine the extent of atherosclerosis in the aortic arch region (from the aortic root up to approximately the first intercostal artery), digital images were analyzed by computer-assisted planimetry using the Sigma Scan Pro v5.0 software (Jandel Scientific, San Rafael, CA). For each animal, the area stained with Oil Red O was divided by total area.

For histological analysis and immunohistochemistry (see below), vessels were paraffin-embedded and mounted in a Microm microtome (Heidelberg, Germany). The region analyzed was approximately between the aortic root and the innominate (brachyocephalic) aorta. Once the three valve cusps were reached, consecutive sections (5 μm thickness) were taken from three regions of the aortic arch separated by ~40 μm. Three cross-sections from each region were stained with May-Grünwald Giemsa (Bio-Optica, Milan, Italy), and images were captured with the digital camera mounted on a Zeiss Axiolab stereomicroscope to quantify the medial and intimal (atheroma) areas. The results for each animal were calculated by averaging all independent values.

Immunohistochemistry in aortic arch cross-sections of fat-fed mice was performed to examine the expression pattern of the macrophage-specific protein Mac-3 (30) and the smooth muscle-specific α-isoform of actin (SMα-actin) (31). Immunocomplexes containing rat monoclonal anti-Mac-3 antibodies (Santa Cruz Biotechnology, sc-19991, 1/100) were detected using a biotin/streptavidin-alkaline phosphatase detection system (Ultra Streptavidin Detection System, Level 2, Signet Laboratories). SMα-actin expression was detected using an alkaline phosphatase-conjugated mouse monoclonal anti-SMα-actin antibody (clone 1A4, Sigma, 1/100). Immunocomplexes were visualized with Fast Red (Sigma).

Measurement of telomere length by quantitative fluorescence in situ hybridization (Q-FISH)

Paraffin-embedded aortic cross-sections were processed and hybridized with a PNA telomeric probe and counterstained with DAPI as described previously (32). Specimens were examined in a Leica fluorescent microscope with a x100/NA 1.0 objective lens. Images were captured with a COHU High Performance CCD camera model 49-12-5000, using a red fluorescence filter (Leica I3-513808) for the Cy3-conjugated telomeric probe and a DAPI fluorescence filter (Leica A-513808) for visualization of nuclei.

Proliferation assays

Splenocytes and bone marrow-derived macrophages were obtained from the pooled tissue from three mice as described previously (28, 33). Cells were plated on 24-well culture dishes at the indicated densities and were pulsed with [^{3}H]thymidine (1 μCi/ml, Amersham). Splenocytes resuspended in RPMI containing 10% FBS/0.55 μM β-mercaptoethanol were plated at a density of 2 x 10^5 cells/well and were stimulated for 48 h with the following mitogens: concanavalin Brazilensis (Conc B, 1.8 μg/ml; gift of Dr J. Calvete), lipopolysaccharide (LPS, 10 μg/ml; Sigma), phorbol 12-myristate-13-acetate (PMA, 20 nM; Sigma), and phytohaemagglutinin (Phyto A, diluted from a 100x stock, Invitrogen). Splenocytes were pulsed with [^{3}H]thymidine during the last 24 h of mitogen stimulation.

Macrophage differentiation was induced by exposing bone marrow suspensions to DMEM containing 20% FBS and 30% L929 cell-conditioned medium (LCM) as a source of macrophage colony-stimulating factor (M-CSF). Macrophages were deprived of M-CSF for 18 h to render cells quiescent. Starved cells were seeded (2x10^5 cells/well) and maintained for 24 h in the presence of [^{3}H]thymidine with or without LCM. [^{3}H]thymidine incorporation assays were performed as described previously (34).

Statistical analysis

Results are reported as means ± SE. In experiments with two groups, differences were evaluated using two-tailed, unpaired Student's *t* test. Analysis involving more than two groups was done using ANOVA and Fisher's post hoc test. (Statview, SAS institute). Statistically significant differences were considered at $P < 0.05$.

RESULTS

Short telomeres protect from diet-induced atherosclerosis in apoE null mice

We sought to examine the development of atherosclerosis in mice deficient for Terc. Notably, the breeding of successive generations of Terc null mice is necessary to reach critically short telomeres and associated phenotypes, such as premature graying of hair, alopecia, impaired capacity for healing of skin wounds, diminished angiogenic potential, and reduced lifespan (18, 27, 28, 35–37). Moreover, the mouse as a species is highly resistant to atherosclerosis (38). Bearing these considerations in mind, we generated for our studies fourth generation animals doubly deficient in Terc and apoE (G4Terc$^{-/-}$apoE$^{-/-}$) and singly deficient in apoE (G4Terc$^{+/+}$apoE$^{-/-}$; see Materials and Methods). The apoE-deficient mouse is an excellent model for studies of genetic and environmental influences on the atherosclerotic process, because it develops spontaneous elevations of total plasma cholesterol and complex atherosclerotic lesions resembling those described in humans, a process that can be accelerated by challenging the mice with a Western-type diet (25, 26, 38).

Previous studies have demonstrated a significant reduction of telomere length in hematopoietic cells of late generation Terc$^{-/-}$ mice (28, 35). We examined aortic cross-sections of 2-month-old mice that had been maintained on control chow to determine by Q-FISH the average telomere length in medial SMCs. These studies revealed significantly shorter telomeres in medial SMCs

from G4Terc$^{-/-}$apoE$^{-/-}$ mice compared with G4Terc$^{+/+}$apoE$^{-/-}$ counterparts with intact telomerase (P<0.001, Fig. 1). To assess the impact of telomere shortening on atherogenesis, 2-month-old mice were challenged for 6 wk with a high-cholesterol, high-fat diet. We first examined whole aortas stained with Oil Red O, which marks in red lipid-laden atherosclerotic lesions. Consistent with numerous studies in apoE null mice, atheromas in both groups of mice predominated within the aortic arch (Fig. 2*A*). Notably, G4Terc$^{-/-}$apoE$^{-/-}$ mice disclosed a significant reduction in the area of atherosclerotic lesions compared with G4Terc$^{+/+}$apoE$^{-/-}$ mice (Fig. 2*A*, P<0.0002). We also quantified in cross-sections of the aortic arch the area of atheroma (intimal lesion) relative to the tunica media (Fig. 2*B* and *C*). Both the intimal area and the intima-to-media ratio were significantly reduced in fat-fed G4Terc$^{-/-}$apoE$^{-/-}$ compared with G4Terc$^{+/+}$apoE$^{-/-}$ mice, thus corroborating our findings in Oil Red O-stained arteries showing reduced atherosclerosis by telomere exhaustion.

Cellular composition of atherosclerotic lesions

Numerous studies in both experimental animals and humans have shown that leukocytes, and in particular lipid-laden macrophages, are the main cellular components of incipient atheromas (5). Chemokines and cytokines secreted by neointimal leukocytes activate the underlying vascular SMCs, which are induced to proliferate and migrate toward the subendothelial space to form fibrous cap lesions, a characteristic of more advanced lesions (5). Immunohistochemistry of the aortic arch in fat-fed G4Terc$^{+/+}$apoE$^{-/-}$ and G4Terc$^{-/-}$apoE$^{-/-}$ mice shows abundance of Mac-3-expressing macrophages within atherosclerotic lesions (Fig. 3*A*, *B*, and *G*) and SMα-actin immunoreactive SMCs in the media (Fig. 3*C*-*F*). SMα-actin-immunoreactivity in fibrous caps was also frequent in the atherosclerotic lesions of G4Terc$^{+/+}$apoE$^{-/-}$ mice (Fig. 3*C*, *E*, and *H*). However, expression of this SMC marker was virtually absent in the atheromas of G4Terc$^{-/-}$apoE$^{-/-}$ mice (Fig. 3*D*, *F*, and *H*). These findings are consistent with the notion that atherosclerotic lesions in fat-fed apoE-mice with short telomeres are less advanced than those of control animals with intact telomerase.

G4Terc$^{-/-}$apoE$^{-/-}$ leukocytes display reduced replicative potential

We next sought to investigate the mechanisms contributing to diminished atherosclerosis by telomere shortening. Because the development of hypercholesterolemia is the main atherogenic stimulus in the apoE null mouse model of atherosclerosis, we first examined this parameter. As shown in Fig. 4, the plasma cholesterol level before and after fat feeding was similar in G4Terc$^{+/+}$apoE$^{-/-}$ and G4Terc$^{-/-}$apoE$^{-/-}$ mice, indicating that diminished atherosclerosis in fat-fed mice with short telomeres is not secondary because of reduced plasma cholesterol content in response to telomere dysfunction.

Abundant proliferation of monocytes/macropages and lymphocytes is thought to contribute to atheroma development (5, 39, 40). Thus, one plausible mechanism to account for the atheroprotective effect of telomere exhaustion in apoE null mice may be the impairment of the proliferative capacity of leukocytes. Of note in this regard, previous studies have shown that hematopoietic cells obtained from late generations of telomerase deficient mice display critically short telomeres and diminished replicative potential (28, 35). Because we carried out our studies in an apoE null background, we sought to investigate whether telomere exhaustion can reduce

the proliferative capacity of apoE-deficient macrophages and lymphocytes. To this end, we isolated splenocytes and bone marrow-derived macrophages from G4Terc$^{+/+}$apoE$^{-/-}$ and G4Terc$^{-/-}$apoE$^{-/-}$ mice to quantify in vitro [^{3}H]thymidine incorporation into replicating DNA. Under basal conditions, cultures of G4Terc$^{-/-}$apoE$^{-/-}$ splenocytes disclosed diminished [^{3}H]thymidine incorporation vs. G4Terc$^{+/+}$apoE$^{-/-}$ cells (Fig. 5, compare controls). Moreover, proliferative responses to B, T, and B+T cell-specific mitogens were significantly reduced in G4Terc$^{-/-}$apoE$^{-/-}$ splenocytes compared with G4Terc$^{+/+}$apoE$^{-/-}$ counterparts (Fig. 5). Likewise, G4Terc$^{-/-}$apoE$^{-/-}$ bone marrow-derived macrophages disclosed reduced proliferation upon stimulation with M-CSF (Fig. 6).

DISCUSSION

By comparing the development of atherosclerosis in fat-fed apoE null mice with an intact Terc gene and with targeted disruption of Terc, the present study demonstrates for the first time that telomere attrition protects from atherogenesis. This atheroprotective effect is not secondary because of reduced hypercholesterolemia in Terc-null compared with Terc-intact apoE null mice. We found that short telomeres impair the proliferation of both lymphocytes and macrophages, an important step in atherosclerosis development (5, 39, 40). Thus, we propose that telomere attrition resulting in replicative immunosenescence may serve as a mechanism for restricting atheroma progression in hypercholesterolemic mice. A detailed analysis of atherosclerotic tissue by double immunostaining experiments using cell type-specific markers in combination with proliferation (i.e., proliferating cell nuclear antigen, Ki67) and apoptosis markers (i.e., TUNEL, bcl-2, caspases-1, -3, and –8, TRAIL and FAS proteins) will be necessary to shed further insight on the mechanisms underlying the atheroprotective effect of telomere exhaustion.

Because telomere shortening is more obvious in highly proliferative somatic cells (1–3), it has been debated whether decreased telomere length in leukocytes of patients with severe coronary atherosclerosis is merely a consequence of increased cell turnover induced by the chronic inflammatory response underlying the atherogenic process or a primary abnormality that renders the organism more susceptible to atherosclerotic risk factors (22, 24, 41). Although it is important to be cautious when considering how findings that are made in mice relate to humans, the atheroprotective effect of telomere attrition reported herein appears to support the first possibility. However, a conclusive answer to this fundamental question must await the results of epidemiological studies to ascertain if newborns with significantly shorter telomeres in blood circulating leukocytes can be identified, and if so whether they are at higher risk of developing coronary atherosclerosis in adulthood independently of known cardiovascular risk factors.

Notably, mice with critically short telomeres are also less predisposed to tumor development (32, 37). Thus, telomere-based replicative senescence appears to play a critical role in the setting of atherosclerosis and cancer in mice. In this regard, it is remarkable that although human aging is associated with telomere erosion in somatic cells (2, 3), both atherosclerosis and cancer are more prevalent in the elderly. These seemingly conflicting findings might be reconciled if one accepts the hypothesis that increasing cellular damage imposed by prolonged exposure to predisposing risk factors and accumulation of multiple mutations over time may ultimately exceed the protective effect of telomere shortening and of other protecting mechanisms. Of interest in this regard, we have recently shown that 4- to 5-yr-old rabbits display significantly smaller

atherosclerotic lesions than their 4- to 5-months-old counterparts, in spite of comparable hypercholesterolemia induced by the same dietary regimen (42).

In summary, the present study implicates telomere exhaustion as a mechanism for restricting atheroma progression in hypercholesterolemic mice, perhaps in part by imposing replicative immunosenescence. Future studies are warranted to investigate whether additional events involved in atheroma initiation and progression are also impaired by telomere exhaustion (i.e., interaction between blood circulating leukocyte and endothelial cells, transendothelial migration of leukocytes, synthesis of cytokines, chemokines, and extracellular matrix proteoglycans, SMC proliferation and migration, apoptosis) and to assess whether genetic and environmental cardiovascular risk factors affect telomerase activity and/or telomere length.

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Fig. 1

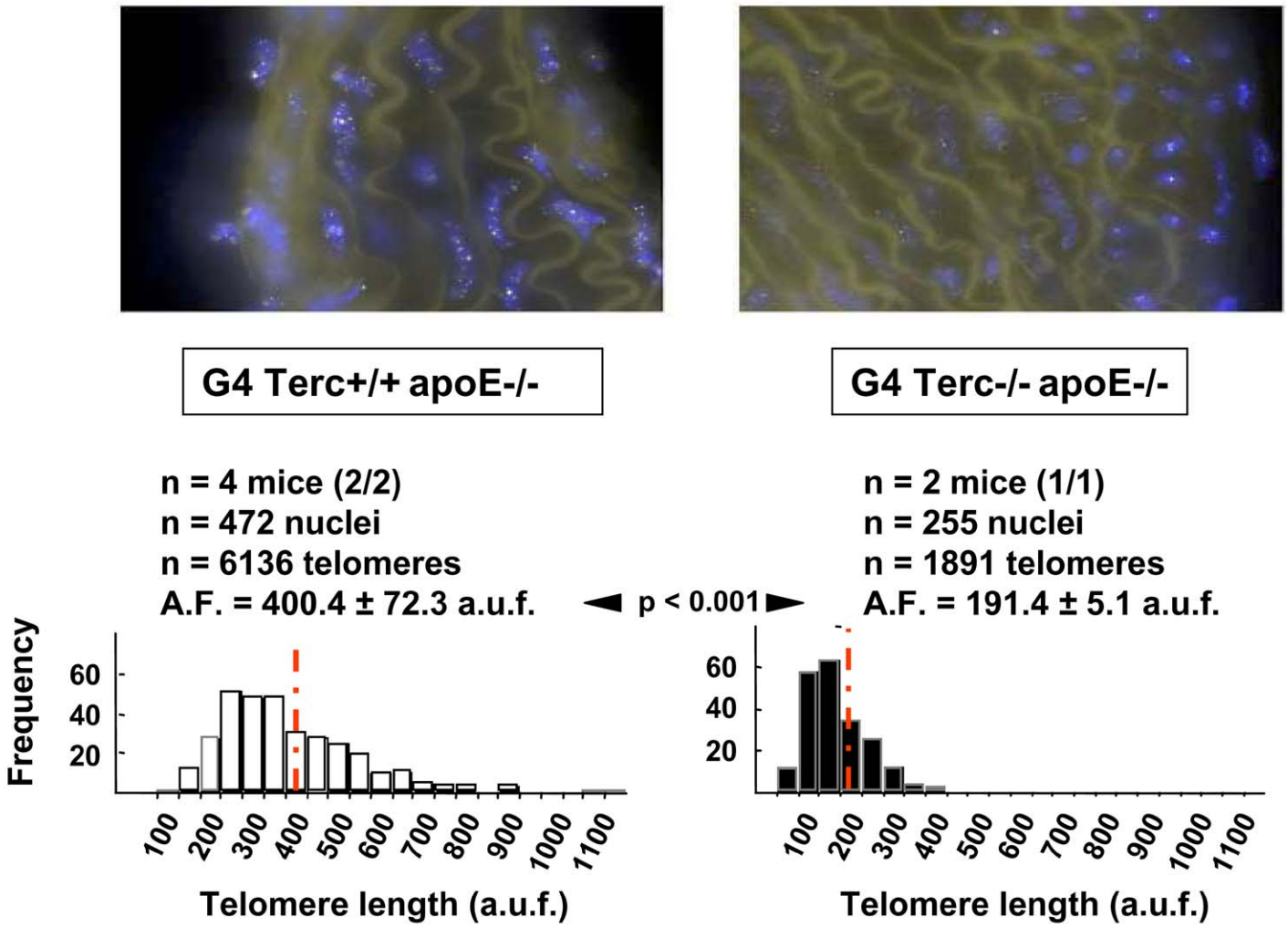

Figure 1. G4Terc$^{-/-}$apoE$^{-/-}$ mice display shorter telomeres than G4Terc$^{+/+}$apoE$^{-/-}$ counterparts. Average telomere length in medial SMCs from the aortic arch of 2-month-old G4Terc$^{+/+}$apoE$^{-/-}$ and G4Terc$^{-/-}$apoE$^{-/-}$ mice fed control chow as determined by Q-FISH using a PNA telomeric probe. Bars in diagrams are the frequency of nuclei with the indicated arbitrary units of fluorescence (a.u.f.). AF: average fluorescence. Number of mice analyzed (*n*) and gender distribution (males/females) are shown. Top photomicrographs are representative examples (green: autofluorescence of elastic fibers; blue: nuclei of medial SMCs; white: telomeric probe). Statistical analysis was performed using 2-tailed, unpaired Student's *t* test.

Fig. 2

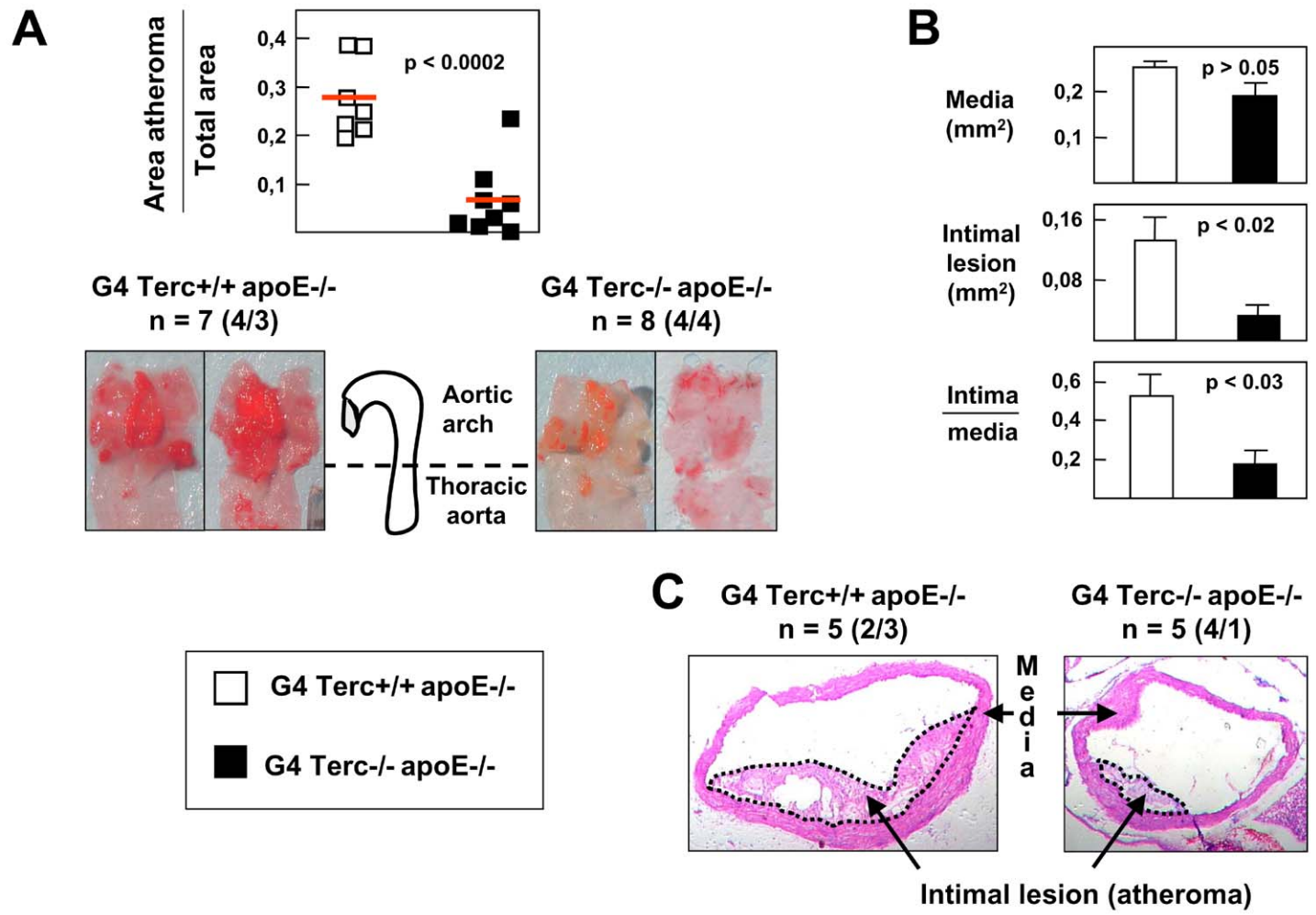

Figure 2. Short telomeres protect from diet-induced atherosclerosis. Two-month-old mice were challenged with a cholesterol-rich diet for 1.5 months. Atherosclerosis within the aortic arch region was quantified by computer-assisted planimetry to determine the area of atherosclerotic lesions after Oil Red O staining in whole-mounted arteries (***A***, lipid-laden lesions in red) and to quantify medial and intimal areas in cross-sections stained with May-Grünwald Giemsa (***B***, ***C***). The edge of the atherosclerotic plaque (intimal lesion) is drawn with a discontinuous line in ***C***. Number of mice analyzed (*n*) and gender distribution (males/females) are shown. Red line in graph in ***A*** is the average value in each group. Statistical analysis was performed using 2-tailed, unpaired Student's *t* test.

Fig. 3

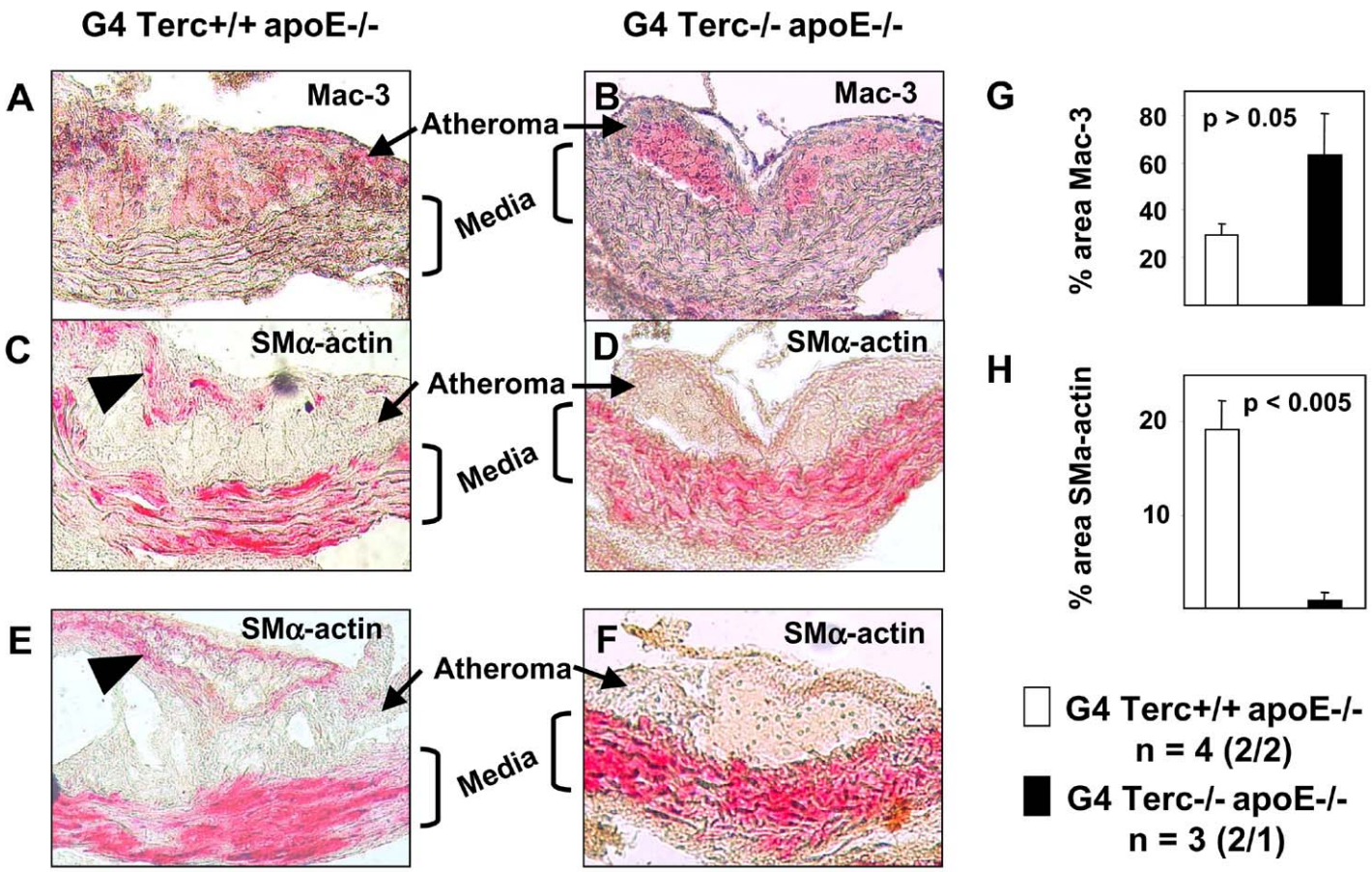

Figure 3. Cellular composition of atherosclerotic lesions. Immunohistochemistry of aortic arch cross-sections of fat-fed mice showing in red Mac-3-immunoreactive macrophages (***A***, ***B***) and SMα-actin-immunoreactive smooth muscle cells (***C***-***F***). Specimens were counterstained with hematoxylin. Top and middle photomicrographs of each genotype show adjacent sections. Arrowheads in ***C*** and ***E*** point at SMα-actin-positive fibrous caps in the lesions of G4Terc$^{+/+}$apoE$^{-/-}$ mice. Atherosclerotic lesions were analyzed by computer-assisted planimetry to calculate the area occupied by Mac-3- and SMα-actin-immunoreactive cells (***G*** and ***H***, respectively). Number of mice analyzed (*n*) and gender distribution (males/females) are shown.

Fig. 4

Figure 4. Reduced atherosclerosis by telomere attrition is not a consequence of diminished hypercholesterolemia. Plasma cholesterol level before and after 1.5 months of fat feeding. Number of mice analyzed (*n*) and gender distribution (males/females) are shown. Statistical analysis was performed using ANOVA and Fisher's post hoc test. Only relevant comparisons are shown. **P* < 0.0001 vs. pre-diet (same genotype); †*P* < 0.05 vs. G4Terc$^{+/+}$ apoE$^{-/-}$ (post-diet).

Fig. 5

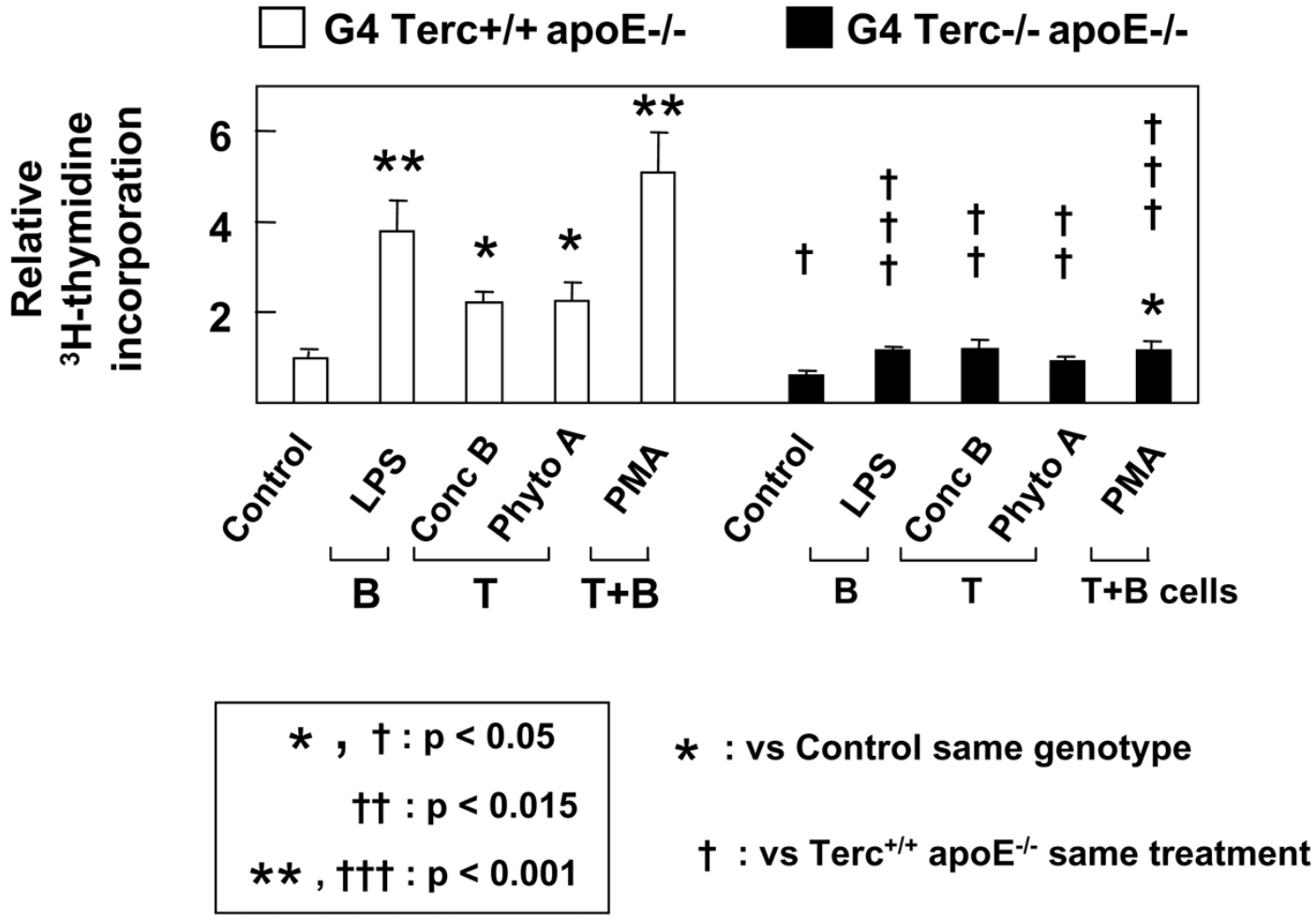

Figure 5. Telomere dysfunction impairs the replicative capacity of apoE null splenocytes. Splenocytes were obtained from the pooled spleens of 3 mice of each genotype. Bars are means ± SE of 4 independent [^{3}H]thymidine incorporation assays. Results are expressed relative to control G4Terc$^{+/+}$apoE$^{-/-}$ cells (=1). Cultures were stimulated for 48 h with the indicated B, T, and B+T cell-specific mitogens. Cells were pulsed with [^{3}H]thymidine during the last 24 h of stimulation. Statistical analysis was performed using ANOVA and Fisher's post hoc test. Only relevant comparisons are shown.

Fig. 6

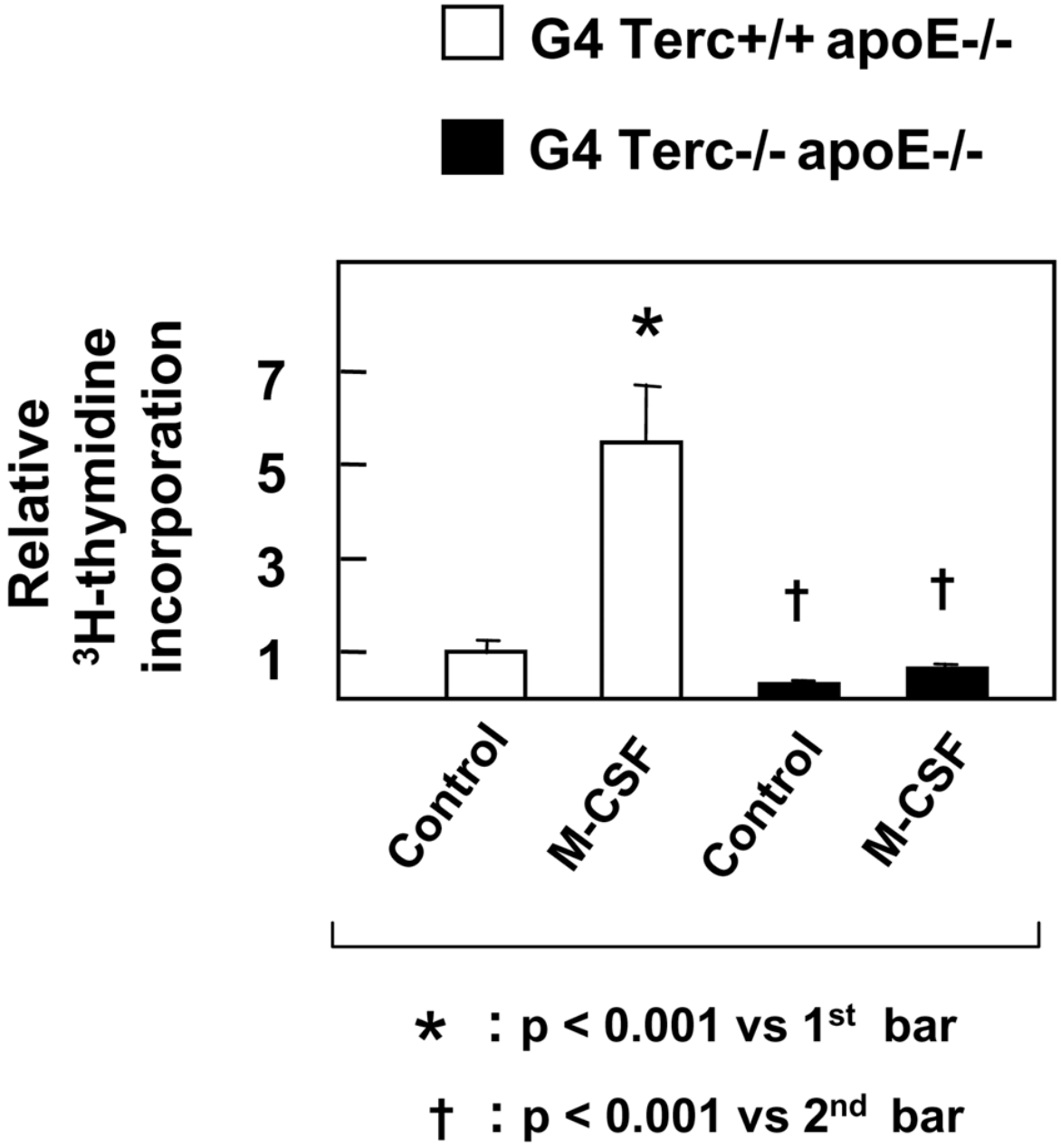

Figure 6. Telomere dysfunction impairs the replicative capacity of apoE null bone marrow-derived macrophages. Bone marrow from 3 mice of each genotype was pooled to obtain macrophages (see Material and Methods). Cultures were pulsed for 24 h with [^{3}H]thymidine in starvation medium (control) or L929 cell-conditioned medium containing M-CSF. Bars are means ± SE of 4 independent [^{3}H]thymidine incorporation assays. Results are expressed relative to control G4Terc$^{+/+}$apoE$^{-/-}$ cells (=1). Statistical analysis was performed using ANOVA and Fisher's post hoc test. Only relevant comparisons are shown.