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Telomere length and replicative aging in human vascular tissues

(atherosclerosis/senescence marker/cell turnover)

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ABSTRACT **Because repeated injury of the endothelium and subsequent turnover of intimal and medial cells have been implicated in atherosclerosis, we examined telomere length, a marker of somatic cell turnover, in cells from these tissues. Telomere lengths were assessed by Southern analysis of terminal restriction fragments (TRFs) generated by *HinfI/Rsa* I digestion of human genomic DNA. Mean TRF length decreased as a function of population doublings in human endothelial cell cultures from umbilical veins, iliac arteries, and iliac veins. When endothelial cells were examined for mean TRF length as a function of donor age, there was a significantly greater rate of decrease for cells from iliac arteries than from iliac veins (102 bp/yr vs. 47 bp/yr, respectively, $P < 0.05$), consistent with higher hemodynamic stress and increased cell turnover in arteries. Moreover, the rate of telomere loss as a function of donor age was greater in the intimal DNA of iliac arteries compared to that of the internal thoracic arteries (147 bp/yr vs. 87 bp/yr, respectively, $P < 0.05$), a region of the arterial tree subject to less hemodynamic stress. This indicates that the effect is not tissue specific. DNA from the medial tissue of the iliac and internal thoracic arteries showed no significant difference in the rates of decrease, suggesting that chronic stress leading to cellular senescence is more pronounced in the intima than in the media. These observations extend the use of telomere size as a marker for the replicative history of cells and are consistent with a role for focal replicative senescence in cardiovascular diseases.**

In the United States and Western Europe, atherosclerosis is the principal contributor to mortality from cardiovascular diseases (1). Prominent among the mechanisms proposed to explain the pathogenesis of atherosclerosis is the "response-to-injury" hypothesis (1–4) in which repeated mechanical, hemodynamic, and/or immunological injury to mural and focal regions of the endothelium is the initiating event to vascular dysfunction. Such insults precipitate an inflammatory–fibroproliferative response from the damaged vasculature. The response is characterized by adhesion of platelets and macrophages on the site of injury, the formation of lipid and cell-rich lesions or "plaques" on the intimal or lumenal surfaces of arterial tissues, and the invasion of underlying smooth muscle cells into the intimal space. If left unchecked, there will follow an age-dependent expansion of the lesion into the lumen, potentially leading to occlusion and infarction at myocardial, cerebral, or other sites (5–7).

A prediction of this hypothesis is that the intimal and medial tissue in the area comprising the atherosclerotic plaque have augmented cell turnover compared to surrounding normal tissue. There is evidence demonstrating age-dependent turnover of vascular tissue. Bierman (8) showed an inverse correlation between donor age and the replicative potential of human arterial smooth-muscle cell culture. Martin *et al.* (9) also showed negative correlations between the number of primary and secondary clones obtained from mouse thoracic smooth muscle cells and donor age, which is consistent with a general decline in replicative potential in cells from medial tissue with age. Moss and Benditt (10) observed that the replicative life-span of cell cultures from arterial plaques was equal to or less than the replicative life-spans from cells of non-plaque areas. In addition, Ross *et al.* (6, 7) showed that cultured smooth muscle cells from fibrous plaques displayed lower responsiveness to whole blood serum when compared to cells from the underlying medial layer. Decreased responsiveness to mitogenic stimuli is characteristic of senescent cells in culture. These results suggest that cells derived from regions of atherosclerotic plaques undergo more cellular divisions than cells from non-plaque areas, rendering the cells in atherosclerotic plaques older and nearer to their maximum replicative capacity. It is also possible that repeated damage to the endothelium may promote the expansion of medial subpopulations whose replicative capacities are greater than those cells occupying the original plaques (11, 12).

Thus, to understand the pathogenesis of atherosclerosis, alterations in cell turnover from tissues implicated in the formation of arterial lesions should be examined. Unfortunately, current markers for the progression of atherosclerosis or for the propensity of an individual to develop atherosclerosis do not directly address this issue. For example, endothelium-derived type III collagen (13), von Willebrand's factor VIII (14), cholesterol, triglycerides, apolipoprotein B (11), lipoprotein(a) (15–17), endothelin (18), and heparin-releasable platelet factor 4 (19) are associated with atherogenesis and can be detected in the plasma but have not been linked directly to cell aging. A similar situation exists for other markers (20–22), including restriction fragment length polymorphisms associated with susceptible individuals (23, 24).

We wished to determine whether the length of telomeric DNA, which has been shown to be a marker of replicative age and division capacity of several types of human somatic cells (25–30), might also serve as a marker in human vascular tissue. Our results show that endothelial cells lose telomeres *in vitro* as a function of replicative age and that telomere loss, *in vivo*, is generally greater in those tissues involved in or susceptible to atherogenesis. These data show that telomere length can be employed to monitor the replicative history of tissues implicated in atherosclerosis and that replicative senescence of vascular cells may play a critical role in atherogenesis.

MATERIALS AND METHODS

Endothelial Cell Cultures. Human umbilical vein endothelial cells were obtained from Thomas Maciag (American Red Cross). Human endothelial cells from iliac arteries and veins were obtained from the Cell Repository of the National Institute of Aging (Camden, NJ). Cells were grown at 37°C in

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Abbreviations: TRF, terminal restriction fragment; PD, population doubling; PDL, PD level.

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5% CO_2 on 100-mm tissue plates whose interiors were treated overnight with 0.4% gelatin (37°C). The supplemented medium consisted of M199, 15% fetal bovine serum, 5 units of heparin per ml, and 20 μg of crude endothelial cell growth supplement (Collaborative Research) or crude endothelial cell growth factor (Boehringer Mannheim) per ml. Cultures were trypsinized (0.05%, 3 min) at confluence, reseeded at 25% of the final cell density, and refed every 2–3 days.

Tissue Samples. Tissue samples from the aortic arch, abdominal aorta, iliac artery, and iliac vein were obtained from autopsies at the Department of Pathology, Health Sciences Center, McMaster University (Hamilton, ON Canada). Within ≈1–2 hr after death, the bodies were stored at 4°C until autopsy, which took place between 4 and 24 hr after death. Control experiments demonstrated that telomere lengths remain unaltered between DNA from vascular tissues left at 4°C for 4 and 24 hr (data not shown). Causes of death are summarized in Table 1. At present it is uncertain how the diseases listed in Table 1 or corresponding treatments might have influenced cell turnover or integrity of telomeric DNA in vascular tissue. Separation of intimal, medial, and adventitial tissue was performed as described (31). However, gross degradation of DNA was not observed from the samples analyzed here. Briefly, the intima was obtained by cutting longitudinally the arteries or veins and carefully scraping off the lumenal surface with a no. 10 scalpel. The isolated intimal material was either treated directly for extraction of DNA or processed for cell culture. To isolate the media, the adventitial layer was removed by cutting or scraping the non-lumenal side of the vessel. Periodic histological examination of tissues confirmed the separation of the intima and media. The medial layer was prepared for DNA extraction by freezing it in liquid N_2 and grinding it in a liquid N_2-chilled mortar and pestle (32). After the tissue was ground to a powder, 5 ml of frozen digestion buffer (10 mM Tris/100 mM NaCl/25 mM EDTA/0.5% SDS, pH 8.0) was added and ground into the powdered tissue. The powder was then incubated at 48°C until thawed. Proteinase K was added to a final concentration of 0.2 mg/ml. After a 12–16 hr of incubation, the solution was either prepared for DNA extraction or stored at −20°C.

Extraction and Restriction Enzyme Digestion of Genomic DNA. DNA was extracted by standard methods as described (26). Extracted, undigested DNA was quantified by fluorometry (33) and then digested simultaneously with *Hin*fI/*Rsa* I (1–3 units/μg of DNA) for 3–24 hr at 37°C. Completeness of digestion as well as the integrity of the DNA before and after digestion were monitored by gel electrophoresis. The terminal restriction fragment (TRF) has previously been shown to contain the telomeric region with several kilobase pairs of the repeat sequence $(TTAGGG)_n$ and a subtelomeric region of non-TTAGGG DNA of variable length (26).

Southern Blot Hybridization. Electrophoresis of digested genomic DNA was performed in 0.5% agarose gels in 45 mM Tris-borate/1 mM EDTA, pH 8.0, for a total of 650–700 V/hr as described (26). After electrophoresis, the gels were placed onto 3 MM Whatman filter paper and dried under vacuum for 25 min at 60°C. Gels were alkaline denatured in 0.5 M NaOH/1.5 M NaCl for 10 min at room temperature and then neutralized in excess 0.5 M Tris/1.5 M NaCl for 5 min. Gels were then immersed in a standard hybridization solution (26) (5× Denhardt's solution/5× SSC/0.1 M Na_2HPO_4/0.01 M $Na_4P_2O_7$, 33 μg of salmon sperm DNA per ml/0.1 μM ATP) that contained the telomeric probe, ^{32}P-labeled $(CCCTAA)_3$ (0.5–1 pmol/ml, 125,000–250,000 cpm/ml). Incubation was for 12–16 hr at 37°C. Finally, gels were washed in 0.24× SSC at room temperature (2 × 5 min). Telomeric smears were visualized through autoradiography on preflashed (OD_{545} = 0.15) Kodak XAR-5 film. The mean lengths of the TRFs were calculated from densitometric scans of the developed films as described (26). Student *t* tests and ANOVA analysis were performed using the STATVIEW 4.0 software package.

Table 1. Postmortem times and causes of death for the autopsy individuals

Age, yr	Sex	Postmortem time, hr	Cause of death
3.5	♀	8.5	Liver failure
14	♀	5.5	Hodgkin disease
19	♀	8	Hodgkin disease
26	♀	8	Graft vs. host disease
35	♀	4	Pulmonary thromboembolism
52	♀	2.5	Leukemia
58	♂	ND	Surgical waste from coronary bypass
74	♀	24	Cardiac insufficiency
74	♀	24	Adenocarcinoma
75	♀	6	Coronary artery disease
77	♂	6.5	Congestive heart failure, sepsis
88	♀	4	Myeloid leukemia
102	♀	4	Bronchopneumonia

ND, not determined.

RESULTS

Loss of Telomeric DNA with Replicative Age in Cultured Endothelial Cells. To determine the applicability of telomere length as a marker for cell turnover in atherosclerosis, we first measured mean TRF length in cultured endothelial cells of the umbilical vein where cell division can be directly monitored *in vitro*. DNA was digested with *Hin*fI and *Rsa* I and the resulting TRFs were subjected to Southern analysis (Fig. 1). Control experiments in which *Hin*fI/*Rsa* I-digested DNA was visualized in ethidium bromide-stained gels demonstrated completeness of digestion. As in human skin fibroblasts (25, 26), mean TRF length decreased as a function of population doublings (PDs). However, the rate of loss of telomeric DNA from endothelial cells (190 ± 10 bp per PD, Fig. 2) was considerably

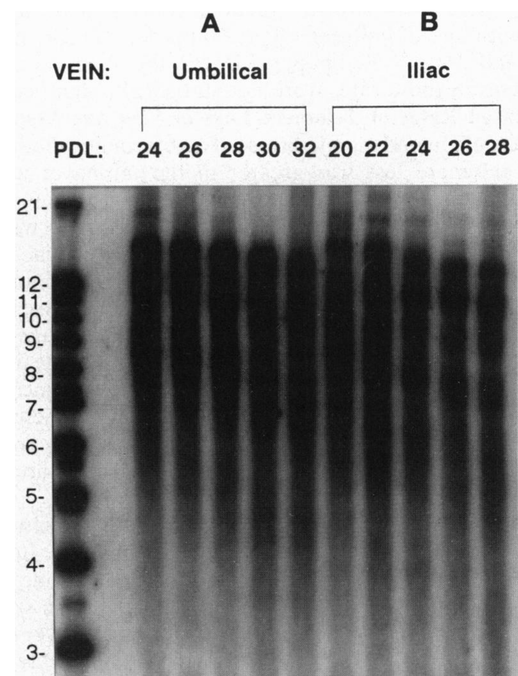

FIG. 1. Representative autoradiogram showing Southern hybridization of telomeric DNA from human endothelial cells of umbilical vein (CH9828) (*A*) or iliac vein (AG11188) (*B*) as a function of population doubling level (PDL). Size markers (kbp) are indicated.

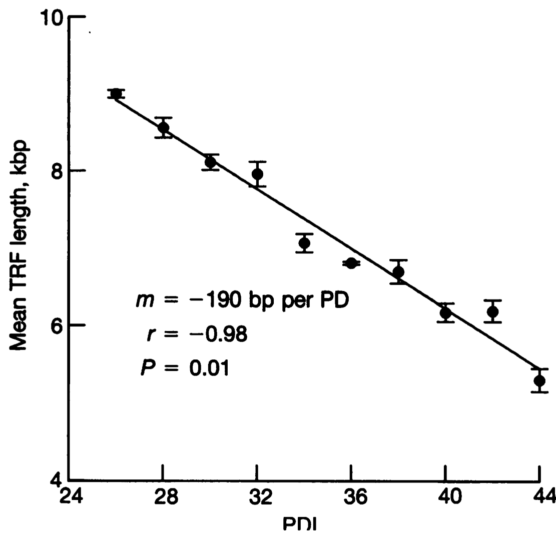

FIG. 2. Linear regression analysis of mean TRF vs. PDL for human umbilical vein endothelial cell cultures. The slope (m), correlation coefficient (r), and P value testing the null hypothesis that the slope is 0 are as indicated. Data points represent the mean of three Southern analyses for each DNA sample.

higher than that attained for human fibroblasts (≈50 bp per PD, 0–91 yr) (25, 26). Variations in the rate of telomere loss may be attributable either to tissue specificity or to several cell culture parameters such as concentration of growth factors, fetal bovine serum, and cell plating efficiency. The y intercept (extrapolated to PDL = 0) is 14.0 ± 0.5 kbp for cells from a neonate, whereas mean TRF length at senescence was 5.7 ± 0.4 kbp. These values are similar to those from human fibroblasts (26).

To determine whether telomere length decreased in endothelial cells from other vascular sources, mean TRF length vs. PDL was determined for several strains of endothelial cells from human iliac artery and vein. A representative autoradiograph is shown in Fig. 1*B*. In iliac arteries and iliac veins there were significant ($P < 0.05$) linear decreases in mean TRF length with age of culture: 140 ± 60 bp per PD for the iliac artery and 160 ± 30 bp per PD for the iliac veins. The differences in these rates were not statistically significant.

Increased Rates of Telomere Loss *in Vivo* Are Associated with Sites of Hemodynamic Stress. To determine whether the rate of telomere loss was greater in the intima of arteries exposed to high hemodynamic stress and hence presumably to greater cell turnover as compared to that of an artery with low hemodynamic stress, we analyzed tissue from the iliac artery and the internal thoracic artery from the same donor. The internal thoracic artery is known to be free of atherosclerotic plaques even among the elderly (51). We found an augmented loss of telomeric DNA in the iliac artery compared to the internal thoracic artery, which became more dramatic with increasing donor age (Fig. 3). Analysis of medial DNA from these same tissues indicated that there was no significant difference in mean TRF length between iliac artery and internal thoracic artery for any donor age studied (data not shown).

Parallel studies were also performed on various strains of endothelial cells from the iliac artery and iliac veins. Because formation of atherosclerotic plaques occurs in the iliac artery rather than in the iliac vein (35) and hemodynamic stress is greater in the former tissue, it is expected that turnover *in vivo* of intimal tissue from the iliac artery should be greater than that from the iliac veins. To test this, nine different strains of endothelial cells from iliac arteries and veins of donors ranging in age from 14 to 58 yr were cultivated and TRF lengths from the earliest possible PDL were determined. Among the nine strains of endothelial cells, there were cultures from the iliac

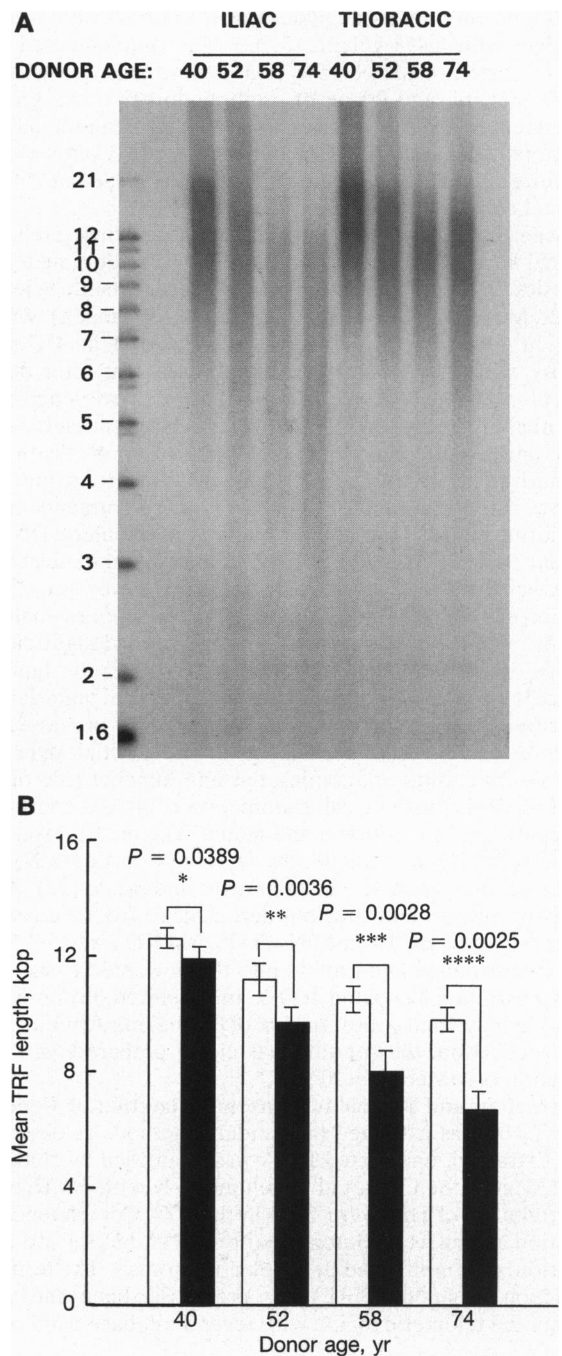

FIG. 3. Autoradiography (*A*) and histogram (*B*) representing decrease in mean TRF length for primary intimal tissue from the internal thoracic artery (□) and iliac artery (■) as a function of donor age. Data for *B* represent mean of three separate Southern analyses. Statistical calculations are based upon the Student's t test. Tissues from same age cohort all originated from same donor. Linear regression parameters for the iliac arteries are $m = -147$ bp/yr, $r = -0.96$, $P = 0.03$ and for internal thoracic arteries are $m = -87$ bp/yr, $r = -0.98$, and $P = 0.06$.

artery and iliac vein from the same individuals for three of the donors, aged 21, 47, and 49 yr. Consistent with the hypothesis of greater cell turnover *in vivo* in arteries than in veins, the rate of decrease in mean TRF length over the age range 20–60 yr for iliac arteries was significantly greater than that for the iliac veins (Table 2).

DISCUSSION

Alterations in cellular turnover of cardiovascular tissue contribute to the many factors leading to cardiovascular diseases.

Table 2. Rates of loss of telomeric DNA as function of donor age for human endothelial cells

Tissue	Age range, yr	n	Rate of loss,* bp/yr	Correlation coefficient
Iliac artery	21–58	4	102 ± 13	0.98
Iliac vein	14–49	5	47 ± 4†	0.71

*Mean ± SD.
†Rate of telomere loss was greater for cells from the iliac artery vs. that from the iliac vein ($P < 0.05$).

For instance, age-dependent hyperplasia and hypertrophy of vascular tissue have been observed in human (34, 35) and nonhuman (32, 36) hypertensives. In atherosclerosis, repeated denudation and subsequent endothelial cell repopulation of the injured endothelium likely contribute to the formation of plaques (2, 3, 6, 7). A useful tool for the field would be a marker that directly monitors cellular turnover of cardiovascular tissue.

Current markers for atherosclerosis include bioassays of the plasma (13–19, 37, 38), the cytoplasm (39), and the cell surface (21). Physiological (22, 40) and genetic (23, 24) markers have also been explored. However, none of the above directly monitors the cell turnover of cardiovascular tissue. Recent investigations have demonstrated the potential of employing telomere length measurements (expressed in terms of mean TRF length) as a marker of replicative history and future replicative capacity of normal somatic cells (25, 26, 29, 30).

We examined the feasibility of using telomere length as a measure of divergent replicative histories for different regions of the human vasculature. For human umbilical vein endothelial cells in culture, telomeres shorten at a rate of ≈190 bp per PDL. We also observed a loss of mean TRF length as a function of PD for several endothelial cell cultures from the iliac artery and the iliac vein, thus showing that mean TRF length serves as a marker for cell turnover *in vitro*. Mean TRF length of endothelial cell cultures from iliac arteries and iliac veins also showed an inverse relationship to donor age, indicating that there is a relationship between donor age and telomere length. This is supported by the observation that mean TRF length also decreased as a function of donor age for primary medial and intimal tissue from the abdominal aorta, aortic arch, and iliac arteries and veins (data not shown), suggesting that a gradual turnover of cells occurs in cardiovascular tissue *in vivo*. The results from the primary tissue are consistent with reports that proliferating cell nuclear antigen-positive (and thus replicatively active) cells are present throughout the rabbit media and intima (41) and with reports that replicative capacities of human smooth-muscle cell cultures decline as a function of donor age (9).

Because telomere loss is correlated with cell turnover *in vitro* and *in vivo* (42), we predicted that vascular tissues with lower rates of cell turnover would possess a higher mean TRF length (due to decreased loss of telomeric DNA per year) than tissues with presumably higher rates of cell turnover. An increased rate of telomere loss as a function of donor age was found in endothelial cells from iliac arteries compared to iliac veins, consistent with the greater propensity of iliac arteries to form atherosclerotic plaques (43). These results suggest that early exhaustion of replicative capacity in endothelial cells from iliac arteries together with known changes in gene expression in senescent cells (44–47) may contribute to augmented plaque formation and thrombosis in iliac arteries. Thus, measurement of telomere length could be a useful procedure to detect alterations of cellular turnover in tissues associated with cardiovascular diseases.

As the intima is the main recipient of hemodynamic insults, we also predicted an augmented loss of telomeric DNA as a function of donor age for specific portions of primary intimal tissue. The increased loss of mean TRF length with respect to donor age in intimal DNA from focal regions of the iliac artery, as compared to intimal tissue from the internal thoracic artery (Fig. 3), likely reflects the increased rate of cell turnover in a region of high hemodynamic stress (iliac artery) over one of low hemodynamic stress (internal thoracic artery). These data are consistent with the comparisons of telomere length in endothelial cells from the iliac artery vs. the iliac vein and demonstrate that the increased rate of telomere loss in the iliac artery is not tissue specific. Telomere length in the media of these same tissues also decreased with age *in vivo* but did not show an increased rate of loss in the iliac artery compared to the internal thoracic artery (not shown). This suggests that the rate of cell turnover overall in the multilayered media is not significantly increased in areas of high hemodynamic stress. It will be of interest to determine whether smooth muscle cells that invade the intima have a reduced telomere length compared to smooth muscle cells in the adjacent media.

If the response-to-injury hypothesis for the pathogenesis of atherosclerotic plaques is correct, then a convenient clinical assay requires a marker of cell turnover for endothelial cells as well as a sampling method that produces minimal damage to the endothelium. The conventional approach of assaying for blood-borne factors that are released from the endothelium has the advantage of noninvasiveness of the intimal layer, but the relationship between plasma concentration of the marker and cell turnover is unclear and likely to be complex. Furthermore, determination of the cellular source of the marker may be difficult. Measurement of telomere length is a direct register of proliferative history, but to obtain telomeric DNA, one must obtain a biopsy of endothelial tissue. Because removal of the endothelium in itself can induce plaque formation, the biopsy strategy obviously entails ethical and practical difficulties. These problems may be minimized through the development of an *in situ* assay or a microassay of telomere length involving <100 cells (unpublished data).

In summary, we have established that mean TRF length can serve as a marker for cell turnover of human vascular tissue. It is likely that not only the loss of replicative capacity but also the alteration in gene expression seen in senescent cells (44–47) contributes to age-related cardiovascular disease. If senescent endothelial cells accumulate at focal sites of high cell turnover, then their reduced ability to divide and form a continuous monolayer will expose the underlying media to blood-derived mitogenic and adhesive factors, which would contribute to the formation of the expanded intimal morphology characteristic of an atherosclerotic plaque. Aberrant expression of genes such as endothelin (48) and intercellular adhesion molecule 1 (49) in senescent endothelial cells could also participate in atherogenesis. It is possible that telomere length or other measures of cellular senescence could predict the functional status of tissues better than chronological age and hence be considered "biomarkers" of aging (50). This knowledge could be employed for the further elucidation of the pathogenesis of cardiovascular diseases and provides for new diagnostic and therapeutic strategies.

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