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Telomere Shortening in Human Coronary Artery Diseases

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Background—Increased cell turnover in response to injury is considered to be important in the development of atherosclerotic plaques. Telomere shortening has been shown to be associated with cell turnover. We assessed the telomere length of human coronary endothelial cells to clarify whether there is a relationship between telomere shortening and coronary artery disease (CAD).

Methods and Results—Coronary endothelial cells were obtained from 11 patients with CAD who underwent autopsy and 22 patients without CAD who underwent autopsy by scraping off the luminal surface of coronary arteries. DNA extracted from the endothelial cells were blotted and hybridized with telomere-specific oligonucleotide ([TTAGGG]_n). The hybridization signal intensity, which represented telomeric DNA content, was standardized with centromeric DNA content (T/C ratio) to estimate telomere length. The T/C ratios were significantly smaller ($P < 0.0001$) in CAD patients than in age-matched non-CAD patients (CAD patients, 0.462 ± 0.135 ; non-CAD patients, 1.002 ± 0.212). In 6 individual CAD patients, the T/C ratio at the atherosclerotic lesion was significantly smaller ($P < 0.05$) than that at the non-atherosclerotic portion.

Conclusions—These findings suggest that focal replicative senescence and telomere shortening of endothelial cells may play a critical role in coronary atherogenesis and CAD. (*Arterioscler Thromb Vasc Biol.* 2004;24:546-550.)

Key Words: telomere ■ senescence ■ endothelial cells ■ coronary artery disease

A close relationship between aging and cardiovascular diseases has been a matter of great interest in the field of cardiovascular and geriatric medicine. Atherosclerosis is a common underlying condition, and aging is considered a major risk factor of atherosclerosis.^{1,2} In addition, aging-related endothelial dysfunction appears to be an important factor that links aging to cardiovascular diseases.³⁻⁶

A growing interest in aging-related changes has been focused on the cellular senescence mechanism. All somatic cells including endothelial cells possess limited replicative potential.^{7,8} If the reserved replicative potential reaches a critical level, then cellular functions are altered or deteriorated. This process is recognized as a fundamental mechanism of cellular senescence.⁹ Accumulating evidence has suggested a pivotal role of telomere shortening in this cellular senescence mechanism. Telomere, which consists of tandem repeats of a short DNA sequence, d(TTAGGG)_n in humans, and associated proteins, is located in each chromosome end.¹⁰ The end-replication problem that DNA polymerases are unable to copy the terminus of a DNA duplex¹¹ causes telomere shortening in proportion to the frequency of cell replication.^{12,13} Concerning telomere length in endothelial cells, it has been revealed that telomeres are shortened as a

function of donor age and are shorter in atherosclerotic lesions of the human abdominal aorta than in non-atherosclerotic portions.¹⁴⁻¹⁶

Ischemic heart disease/coronary artery disease (CAD) is a representative atherosclerosis-associated disorder. Cellular senescence and endothelial dysfunction were suggested to be important in its pathological mechanism.¹⁷ Although telomere shortening in leukocytes of patients with CAD has been reported,¹⁸ telomere length of coronary endothelial cells of such CAD patients has not been directly measured. Because of the small size of coronary arteries, it is difficult to obtain an adequate quantity of DNA from coronary endothelial cells for telomere length measurement using Southern hybridization analysis.

In the present study, we assessed telomere shortening in coronary endothelial cells of patients with CAD using a recent technique, slot blot analysis, by which the relative telomere length could be evaluated in a small amount of DNA. The results were compared with those from subjects with neither coronary atherosclerosis nor CAD. We also compared the telomere length of coronary endothelial cells between the atherosclerotic site and the non-atherosclerotic site in the same individual.

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Methods

Tissue Samples

In this study, coronary arteries were obtained at autopsy at our hospital from 13 patients with CAD and 35 patients without CAD. All these autopsies were performed within 3 hours after death, and the coronary arteries were dissected from the epicardial surface in a fresh state. From each patient, one segment (25 to 50 mm in length) of the coronary artery was sampled. In the patients with CAD, coronary segments containing the culprit lesion responsible for acute or old myocardial infarction were excluded. Then, the proximal part of each coronary segment was sliced into 3 pieces serially at approximately 5-mm intervals. Proximal and distal slices were fixed in methanol-Carnoy fixative and embedded in paraffin blocks for histological investigations, and a mid-portion slice was snap-frozen at -80°C until DNA preparation (see later). Serial sections were cut from each paraffin block and stained with hematoxylin and eosin and Weigert elastic van Gieson stain to test whether the frozen mid-portion sample for DNA preparation contained an atherosclerotic lesion. After light microscopic investigation of these sections, 22 sites containing no atherosclerotic lesion were selected as control from the patients without CAD, and 11 sites containing a distinct atherosclerotic lesion were selected from the patients with CAD. In this selection, sites with ruptured or eroded plaques, characterized by rupturing of the fibrous cap or superficial erosion, were excluded, because these ruptured or eroded lesions appeared to be unsuitable to detect adequate telomeric DNA content.

In 10 of the 13 patients with CAD, 3 additional 5-mm serial slices were obtained from the distal part of each coronary segment sampled and were used for a comparative examination between the proximal atherosclerotic site and the distal non-atherosclerotic site in the same individual. Serial sections were cut and stained in a similar fashion, and after light microscopic investigation, 6 distal sites containing no atherosclerotic lesion were selected for this comparative study.

As a result of the case selection, 22 frozen coronary samples containing no atherosclerotic lesion from 22 patients without CAD (16 men and 6 women; age 47 ± 22 years) and 11 frozen coronary samples with a distinct atherosclerotic lesion from 11 patients with CAD (9 men and 2 women; age 65 ± 11 years) were subjected to further study of DNA preparation. In addition, 6 frozen coronary samples without an atherosclerotic lesion obtained from the CAD patients were also subjected to the comparative study.

Clinical characteristics of the 11 patients with CAD, including age, gender, types of CAD, cause of death, and presence of risk factors (cigarette smoking, hypertension as defined by the Joint National Committee V,¹⁹ diabetes mellitus as defined by the WHO Study Group,²⁰ and hypercholesterolemia; cholesterol level >220 mg/dL) are summarized in Table I (available online at <http://atvb.ahajournals.org>). Among the 22 patients without CAD, none had hypertension, diabetes mellitus, or hypercholesterolemia, and 7 were smokers. Informed consents were obtained from the family of the patients who underwent autopsy, and this study was approved by the hospital ethical committee.

Isolation of Coronary Endothelial Cells and Validation Using Immunohistochemistry

Isolation of the coronary endothelial cells was performed according to techniques previously reported.^{14,21} Briefly, each of the frozen coronary artery samples was cut longitudinally, and its luminal surface with endothelial cells was scraped carefully with a scalpel. The isolated endothelial cells were treated directly for extraction of genomic DNA.

To validate the isolation of coronary endothelial cells, immunohistochemical analysis was performed. Frozen sections (6- μm -thick) of scraped coronary artery specimens and a part of isolated luminal surface materials were fixed in acetone for 10 minutes at -4°C and hydrated. Sections were then pretreated with 0.3% H_2O_2 /phosphate-buffered saline for 10 minutes at room temperature and incubated with a blocking solution (phosphate-buffered saline containing 10% normal goat serum) for 30 minutes at room temperature. Sections were incubated with the primary antibody against endothelial cell

von Willebrand factor (Dako) for 1 hour at room temperature, followed by incubation with Envision+ solution (Dako) for 1 hour at room temperature. Peroxidase activity was detected using 3-amino-9-ethyl-carbazole liquid substrate (Dako) as chromogen. Between all steps, tissue sections were rinsed with phosphate-buffered saline. Finally, sections were counterstained with Mayer hematoxylin before mounting.

Extraction of Genomic DNA From Coronary Endothelial Cells

The tissue samples were lysed by incubation at 55°C for 48 hours in 200 μL of lysis buffer containing 10 mmol/L of Tris/HCl (pH 8.0), 0.1 mmol/L of EDTA (pH 8.0), 2% sodium dodecyl sulfate, and 500 $\mu\text{g}/\text{mL}$ of protease K (Roche Diagnostic, Tokyo, Japan). Genomic DNA extraction from coronary artery intimal material was performed using DNeasy Tissue Kit (Qiagen K.K., Tokyo, Japan) according to the manufacturer's recommendations.

Cultured Fibroblast DNA

To validate the specificity of slot blot analysis, we additionally performed control studies using DNA samples extracted from young (16 population-doubling level [PDL]) and senescent (40 PDL) cultured human fibroblasts (TIG-7; Immuno-Biological Laboratories, Fujioka, Japan).

Slot Blot Analysis

The length of telomeric DNA was estimated as the telomeric/centromeric DNA contents ratio (T/C ratio). Because the centromeric DNA contents may be assumed to be essentially identical in all non-transformed cells, the telomeric DNA contents can be standardized by calculating the relative telomeric DNA content with centromeric DNA content. To evaluate the T/C ratio, we performed slot blot analysis as previously reported,²² with minor modification. Briefly, DNA samples (12.5 to 400 ng) were diluted with twice the volumes of transfer buffer solution (0.5 mol/L NaOH, 1.5 mol/L NaCl) and denatured at 55°C for 30 minutes. The slot blot apparatus (The Convertible Filtration Manifold System; Life Technologies, Tokyo, Japan) was assembled according to the manufacturer's instruction, and nylon membranes (Roche) were set in this apparatus. Slot blot wells were washed with transfer buffer solution, and the DNA samples were then loaded for 10 minutes. DNA was cross-linked to the membrane with ultraviolet light (120 000 $\mu\text{J}/\text{cm}^2$). The membrane was incubated with 2 pmol 5'-digoxigenin (DIG)-labeled telomere-specific oligonucleotide [TTAGGG]_n,¹⁰ in 20 mL of DIG Easy Hyb (Roche) for 12 hours at 42°C . After the hybridization step, the membrane was washed in saline-sodium citrate (twice in $2 \times$ saline-sodium citrate at 25°C for 5 minutes and twice in $0.1 \times$ saline-sodium citrate at 42°C for 15 minutes). Hybridization signal of the DIG-labeled probe was converted into chemiluminescent signal using DIG Wash and Block Buffer Set and DIG Luminescent Detection Kit (Roche) according to the manufacturer's recommendations. The chemiluminescent signal intensities were computerized with a digital image captor (LAS-1000 plus; Fuji Photo Film, Tokyo, Japan) and quantified with an image analysis software (Image Gauge ver. 3.2; Fuji Photo Film). After stripping the telomere probe, the membrane was re-hybridized with 2 pmol 5'-DIG-labeled centromere-specific oligonucleotide [GTT TTG AAA CAC TCT TTT TGT AGA ATC TGC]₂₃ at the same conditions. After hybridization washing, chemiluminescence capture and signal intensity quantification were performed again using the same method as described. Absence of significant signal reduction after stripping first probe was confirmed by a preliminary study of repeated hybridization for centromere.

Statistics

Data are expressed as mean \pm SD. Either Mann-Whitney *U* test or Wilcoxon signed-rank test was appropriately used to examine the differences between the two groups. The least-square regression method was used to obtain a simple linear regression equation to

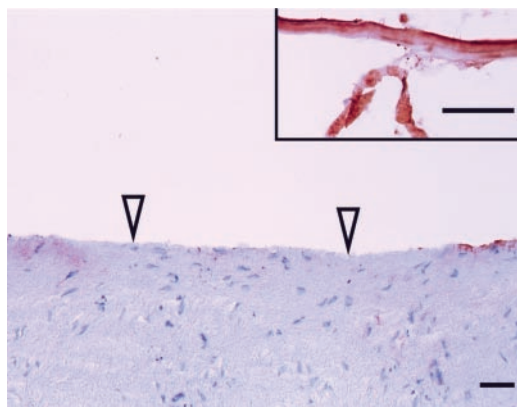


Figure 1. Immunohistochemical staining for von Willebrand factor. A coronary artery specimen after separation of the luminal surface by scraping shows no endothelial cell lining at the luminal surface site (arrowheads). Inset: Coronary artery endothelial cells isolated by scraping. Scale bar=50 μ m.

assess age-related T/C ratio reduction. A level of $P < 0.05$ was accepted as statistically significant.

Results

Validation of the Isolation of Coronary Endothelial Cells

Figure 1 shows a representative result of multiple scrapings from CAD and non-CAD patients. Immunohistochemical staining for endothelial cell von Willebrand factor revealed that endothelial cells were removed from the coronary artery by scraping, and its scraped luminal surface material contained linear endothelial cells (Figure 1, inset).

Slot Blot Analysis: Cultured Human Fibroblasts

Using DNAs extracted from cultured human fibroblasts, we examined the difference in telomere length between young and senescent cultured cells (Figure 2); 100 ng of the DNA sample yielded distinct hybridization signals for telomere and centromere. Although the signal for telomere was weaker in the senescent cells (40 PDL) than in the young cells (16 PDL), the signal for centromere was not different between them. The measurement was performed in triplicate and revealed that the T/C ratio was higher in 16 PDL cells (1.089 ± 0.096) than in 40 PDL cells (0.708 ± 0.103 , $P < 0.001$).

Slot Blot Analysis: Coronary Endothelial Cells

We tested the usability of slot blot analysis in the measurement of telomere length of coronary endothelial cells. Signal

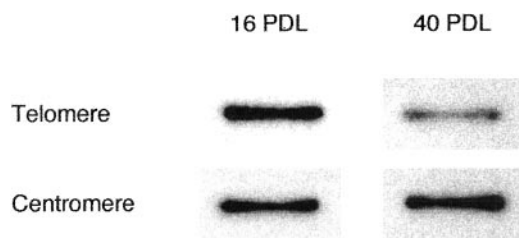


Figure 2. T/C ratio measurement in cultured fibroblasts. Upper and lower panels show signals for telomere and centromere, respectively. The signal for telomere is weaker in the senescent cells (40 PDL; right lane) than in the young cells (16 PDL; left lane), whereas the signal for centromere is not different between them.

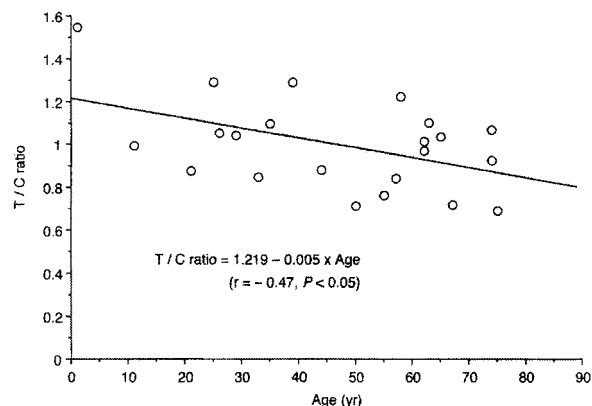


Figure 3. Age-related T/C ratio reduction in patients without CAD. There is a significant ($P < 0.05$) correlation between the T/C ratio and donor age.

intensities for telomeric and centromeric DNA in a genomic DNA sample extracted from the coronary artery endothelial cells with CAD (Figure 1A, available online at <http://atvb.ahajournals.org>) showed a strong correlation to the amount of genomic DNA loaded (Figure 1B). The T/C ratio was almost constant when more than 25 ng of genomic DNA was analyzed (Figure 1C). The stability was also obtained in a genomic DNA from the coronary artery endothelial cells without CAD (not shown).

T/C Ratios and Their Reduction With Aging in Non-CAD Patients

The T/C ratios of coronary endothelial cells ranged from 0.694 to 1.549 in patients without CAD and from 0.238 to 0.747 in patients with CAD. As shown in Figure 3, there was a significant correlation ($r = -0.47$; $P < 0.05$) between the T/C ratio and donor age in the patients without CAD.

Difference in the T/C Ratio Between CAD Patients and Non-CAD Patients

Because the T/C ratios showed age-dependent reduction, we selected 13 of 22 non-CAD patients who were older than 40 years old for age-matched comparison with the patients with CAD. Although the signal intensity for centromeric DNA was not different between the patients with CAD and the age-matched patients without CAD, reduced signals for telomeric DNA were observed in the patients with CAD (Figure 4A). The T/C ratio was significantly smaller ($P < 0.0001$) in the patients with CAD (0.462 ± 0.135) than in the 13 age-matched patients without CAD (1.002 ± 0.212) (Figure 4B).

Difference in the T/C Ratio Between Atherosclerotic Sites and Non-Atherosclerotic Sites of Patients With CAD

In the 6 individual patients with CAD, the T/C ratios of coronary artery sites with and without atherosclerosis were compared. The coronary artery sites with atherosclerosis showed smaller T/C ratios than those without atherosclerosis ($P < 0.05$) (Figure 5).

Discussion

Despite increasing evidence of cellular senescence in patients with CAD,¹⁸ no comprehensive study examining telomere

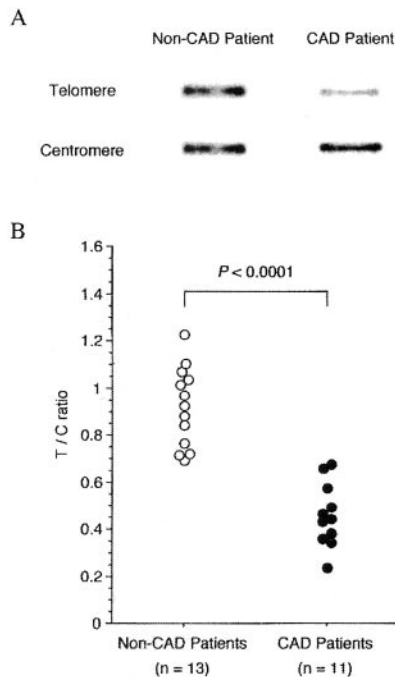


Figure 4. Comparison of the T/C ratio between CAD patients (aged 65 ± 11 years) and age-matched non-CAD patients (aged 62 ± 9 years). A, The signal intensity for telomeric DNA is distinctly more reduced in a CAD patient than in a non-CAD patient, whereas the signal intensity for centromeric DNA is not different between them. B, The T/C ratio is significantly higher ($P < 0.0001$) in the patients without CAD (1.002 ± 0.212) than in the patients with CAD (0.462 ± 0.135).

length of coronary endothelial cells has been reported. Only a few studies have dealt with the measurement of telomere length in the iliac artery or abdominal aorta.^{14–16} These previous studies revealed that the telomere length of endothelial cells was shortened with aging. The present results that the T/C ratios of coronary endothelial cells were reduced as a function of donor age are compatible with these previous

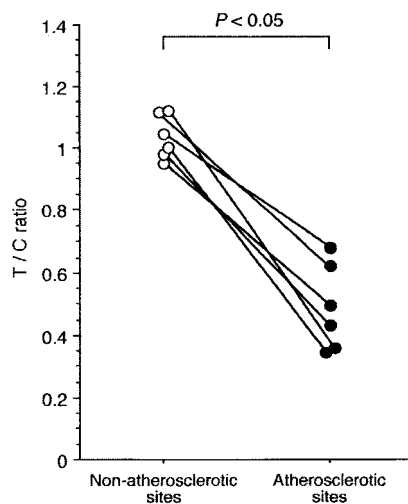


Figure 5. Difference in the T/C ratio of coronary endothelial cells between the atherosclerotic site and non-atherosclerotic site of 6 individual patients with CAD. The T/C ratios are significantly reduced at coronary artery sites with atherosclerosis compared with those without atherosclerosis ($P < 0.05$).

findings. Moreover, several studies have suggested that aging induces endothelial dysfunction, which is recognized to be a factor in cardiovascular diseases.^{3–6} Because telomere shortening and endothelial dysfunction are age-related phenomena, telomere shortening in coronary endothelial cells may be related directly to coronary endothelial dysfunction. Recently, Minamino et al¹⁷ examined coronary arteries obtained from 4 patients with CAD who underwent autopsy using β -galactosidase histochemical staining and found that the coronary endothelial cells were senescent and functionally deteriorated. They also demonstrated that the functions of senescent endothelial cells could be recovered by induction of telomerase, which was a specific enzyme for the telomere elongation. The present findings of the direct measurement of telomere contents in coronary endothelial cells complement their findings and support the concept that telomere shortening in coronary endothelial cells with aging may contribute to coronary endothelial dysfunction and the development of CAD in humans.

The present T/C ratio analysis showed that telomeres of coronary endothelial cells were shorter in patients with CAD than in the age-matched patients without CAD, suggesting that additional factors in telomere shortening other than aging had been acting on patients with CAD. Two mechanisms of telomere reduction have been proposed: (1) human telomeres undergo progressive shortening with cell division through replication-dependent sequence loss at DNA termini;^{11–13} and (2) persistent mild oxidative stress leads to telomere shortening.²⁴ In the present study, most patients with CAD had one or more CAD risk factors. CAD risk factors can become causes of oxidative stress and endothelial injury. Free radicals generated by hypertension,^{25,26} diabetes mellitus,^{27,28} and cigarette smoking^{29,30} can injure endothelial cells. Elevated serum levels of low-density lipoprotein may increase the possibility of the generation of oxidized products, which is a known pathological factor in endothelial injury.^{31,32} These CAD risk factors may contribute to telomere shortening in coronary endothelial cells. In fact, in vitro studies revealed that telomere shortening in endothelial cells could be accelerated by oxidative stress and could be slowed down by suppression of oxidative stress.^{33,34}

We further disclosed that coronary endothelial cells at an atherosclerotic site had reduced T/C ratios compared with those at a non-atherosclerotic site in 6 individual patients with CAD. The difference in T/C ratios of coronary endothelial cells between the atherosclerotic and non-atherosclerotic sites in CAD patients was comparable to that between age-matched patients with CAD and without CAD. These findings suggest that telomere shortening associated with atherosclerosis is primarily a local event rather than systemic phenomena including the congenital shortening. In recent studies of telomere length measurement in human endothelial cells by Southern blot analysis, Chang et al¹⁴ and Okuda et al¹⁶ found that telomere shortening was markedly accelerated in the regions easily affected by atherosclerosis. They suggested that this was caused by the high cellular turnover in the regions induced by local factors such as shear stress. Focally enhanced cellular turnover may cause early cellular senescence associated with telomere shortening and may participate in coronary atherogenesis.

Although Southern blot analysis of terminal restriction fragment lengths is the standard and currently most reliable

method for evaluating telomere length, there are 3 defects in this method. First, terminal restriction fragments contain DNA other than uniform telomeric repeats.³⁵ Second, this analysis requires more than several micrograms of high-molecular-weight genomic DNA. Third, DNA breakage reduces the observable telomere length. Alternatively, slot blot analysis can be performed on small amounts (<20 ng) of sample DNA as was performed in the present study. Moreover, the T/C ratio analysis that we adopted can detect alterations in telomeric DNA content independent of DNA breakage, DNA ploidy, and DNA sample size.²²

The main limitation of the present study was the possible incompleteness in separation of endothelial cells from the coronary arteries. In addition to endothelial cells, a small amount of extracellular matrix and a few smooth muscle cells might have contaminated in the isolated luminal surface materials. However, we confirmed using immunohistochemical staining for von Willebrand factor that the majority (at least 90%) of components of the materials was endothelial cells. Therefore, this suggests that the T/C ratios were of endothelial cells. In situ telomere length measurement recently described by O'Sullivan et al³⁶ may provide a complete solution to the problem.

In conclusion, we revealed that telomeres of endothelial cells implicated in coronary atherosclerosis were markedly shortened. This finding suggests that telomere shortening and replicative cellular senescence in coronary endothelial cells are focal phenomena and may play pathogenic roles in coronary atherogenesis and CAD.

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