The Role of Vascular Cell Senescence in Atherosclerosis

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Abstract: The functional changes associated with cellular senescence may be involved in human aging and age-related vascular disorders. We have shown the important role of telomere and telomerase in vascular cell senescence *in vitro*. Progressive telomere shortening has been observed in atherosclerosis, implicating its contributions to atherogenesis. To investigate whether senescent vascular cells are present in the vasculature, senescence-associated β-galactosidase (β-gal) activity was examined in the coronary arteries and the internal mammary arteries retrieved from autopsied individuals who had ischemic heart disease. Strong β-gal stainings were observed in atherosclerotic lesions of the coronary arteries but not in the internal mammary arteries. An immunohistochemical analysis demonstrated that β-gal stained cells are vascular endothelial cells. To determine whether endothelial cell senescence causes endothelial dysfunction, we induced senescence in human aortic endothelial cells (HAECs) by inhibiting telomere function and examined the expression of intercellular adhesion molecule (ICAM)-1 and endothelial nitric oxide synthase (eNOS) activity. Senescent HAECs exhibited increased ICAM-1 expression and decreased eNOS activity, both of which are alterations implicated in atherogenesis. In contrast, introduction of telomerase significantly extended the life span and inhibited the functional alterations associated with senescence. Our data suggest that endothelial cell senescence induced by telomere shortening contributes to atherogenesis. (J. Jpn. Coll. Angiol., 2003; **43**: 41-46)

Key words： Telomere, Senescence, Aging

Introduction

Cellular senescence is a limited ability of primary human cells to divide when cultured *in vitro*. This cessation of cell division is accompanied by a specific set of changes in cell function, morphology and gene expression. These changes in cell phenotype may contribute to age-associated diseases including atherosclerosis. However, cellular senescence has largely been investigated *in vitro* and the presence of senescent vascular cells *in vivo* has not been clarified.

Recently, accumulating evidence has suggested a critical role of telomere and telomerase in cellular senescence *in vitro*.[1] We have demonstrated previously that introduction of telomerase catalytic component (TERT) into human vascular smooth muscle cells extends cell life span and preserves a younger phenotype, suggesting that telomere stabilization is important for long-term cell viability of vascular cells.[2] Progressive telomere shortening in human arteries has been observed in the regions susceptible to atherosclerosis.[3] Moreover, telomere length has been reported to inversely correlate with pulse pressure and atherosclerotic grade in human.[4, 5] Although these observations imply that telomere shortening *in vivo* may contribute to the pathogenesis of age-associated vascular disorders, it remains unclear whether loss of telomere function induces vascular dysfunction associated with aging.

In the present study, we demonstrate the presence of vascular endothelial cells with senescence-associated phenotypes in the atherosclerotic regions of human coronary arteries. We also show that loss of telomere function induces endothelial dysfunctions that are observed in aged arteries, whereas inhibition of telomere shortening suppresses these alterations with senescence.

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Methods

Tissue specimens

Human coronary arteries and internal mammary arteries were obtained from four autopsied individuals who had ischemic heart diseases. The autopsy tissues were obtained within 12 hours after death and subjected to β-galactosidase (β-gal) staining.

β-galactosidase staining

The autopsy tissues were washed twice in phosphate-buffered saline. After washes, senescence-associated β-gal activity was examined in the tissues as described previously.[6] Briefly, the samples were incubated for 24 hours at 37°C in freshly prepared β-gal staining solutions containing 1mg/mL 5-bromo-4-chrolo-3-indlyl β-D-galactopylanoside (X-gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium fericyanide, 150 mmol/L NaCl, 2 mmol/L $MgCl_2$, 0.01% sodium deoxycholate and 0.02 % Nonidet-40. After the stained arteries were photographed, the samples were immersed in OCT compounds (Miles Inc.) and snap-frozen in liquid nitrogen to prepare cryostat sections.

Immunohistochemistry

The frozen sections (6 μm) were treated with 0.3 % hydrogen peroxide in methanol for 20 min, preincubated with 5 % goat serum and then treated with the anti-α-smooth muscle actin antibody for vascular smooth muscle cells (1: 50, Pharmingen), anti-CD68 antibody for macrophages (1: 50, DAKO) and anti-factor VIII antibody for endothelial cells (1:100, DAKO) for 1 hour at 37°C. Next, the sections were incubated with a biotinylated goat secondary antibody, treated with the avidin-biotin complex (Elite ABC kit, Vector) and stained with diaminobenzidine tetrahydrochloride and hydrogen peroxide.

Retroviral infection

The expression vector of a dominant-negative form of human TRF2 with FLAG tag (TRF2D/N), pTetFLAGhTRF2$^{44\text{-}454}$, was the kind gift of Dr. de Lange (Rockefeller University, New York, NY). TRF2D/N was then cloned into a pLPCX retroviral vector (Clonetech). Retroviral stocks were generated as previously described.[2]

Western blot

Whole-cell lysates were resolved on 10% SDS-PAGE. Proteins were transferred onto a PVDF membrane (Millipore) and incubated with anti-ICAM-1 antibody, anti-endothelial nitric oxide synthase (NOS) antibody (Santa Cruz) or anti-FLAG antibody M2 followed by an anti-rabbit IgG horseradish peroxidase antibody or anti-mouse IgG horseradish peroxidase antibody (Jackson). Specific proteins were detected using ECL (Amersham).

Southern blot analysis

Genomic DNA (2 μg) extracted by standard methods was digested with Hinf I / Rsa I and resolved in 0.5 % agarose gels. The blot was then hybridized with ^{32}P-labeled $(CCCTAA)_3$ at 42°C overnight to detect telomeric DNA, washed at highly stringent condition with 0.1x SSC-0.1% SDS and analyzed with a phosphorimage analyzer (Molecular Dynamics).

NOS activity assay

The NOS activity was examined with NOS assay kit (Calbiochem) according to manufacturer's instructions.

Statistical analysis

All values were expressed as mean ± SEM. Comparison of results between different groups was performed by one way analysis of variance or paired *t*-test using StatView 4.5 (Abacus Concepts).

Results

Senescent endothelial cell in human coronary arteries

We first asked whether there are senescent vascular cells in human atherosclerotic lesions. We examined senescence-associated β-gal activity in the coronary arteries obtained from 4 autopsied individuals who had ischemic heart diseases. Strong β-gal stainings (blue color) were observed in the coronary arteries but not in the internal mammary arteries from the same patients where atherosclerotic changes were minimally observed (**Fig. 1A**). The cross-sections of arteries stained with β-gal indicated that β-gal-positive cells were mostly located on the luminal surface (**Fig. 1B left**). The high-magnification photograph in **Fig. 1B (right)** demon-

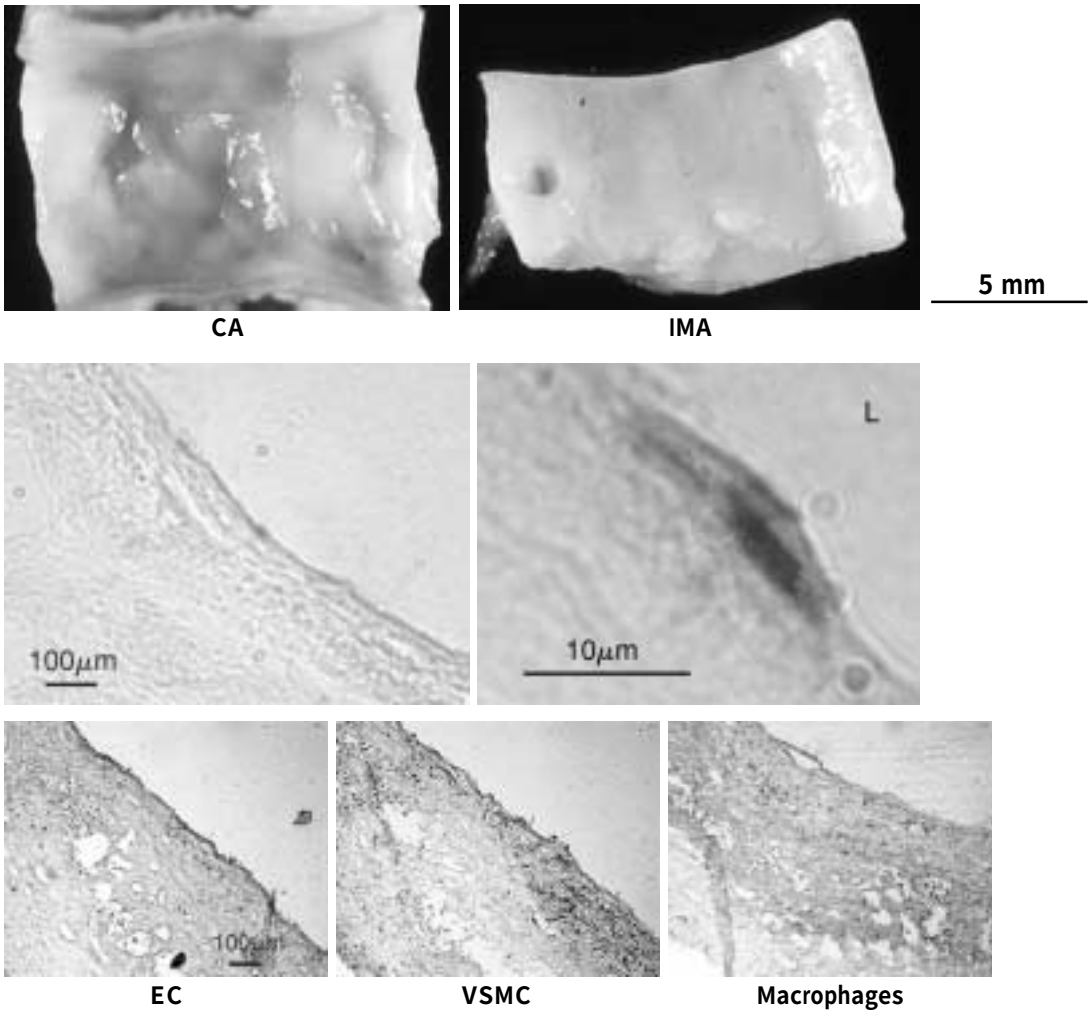

Figure 1 A, Photographs of the luminal surface of human coronary artery (**CA**, left) and internal mammary artery (**IMA**, right) stained with β-gal staining. Senescent-associated β-gal activity was observed in human coronary arteries but not in internal mammary arteries. Similar results were obtained from 4 autopsy specimens. B, The cross-section of the coronary artery in (A) minimally stained with hematoxylin illustrates β-gal-positive cells on the luminal surface (left). The high-magnification picture (right) demonstrates granular blue stainings in the cytoplasm. L: lumen. Original magnification was X200 for left, X1000 for right. C, Immunohistochemistry for factor VIII (**EC**, left), α-smooth muscle actin (**VSMC**, middle) and CD68 (**Macrophages**, right) of serial sections. Original magnification was X100.

strated granular blue stainings in the cytoplasm of the cells on the luminal surface, indicating that the blue stainings are originated from cells, not from extracellular matrix. An immunostaining for factor VIII of the section adjacent to that in Figure 1B confirmed that β-gal-positive cells were vascular endothelial cells (**Fig. 1C**). Immunostainings for α-smooth muscle actin and CD68 showed a typical fibrous plaque formation composed of smooth muscle cell layers and the accumulation of macrophages. β-gal-positive endothelial cells appeared flattened and enlarged in contrast to round shape of endothelial cells in non-atherosclerotic lesions. They were predominately localized on the surface of atherosclerotic plaques, suggesting that vascular endothelial cell senescence may be involved in atherogenesis.

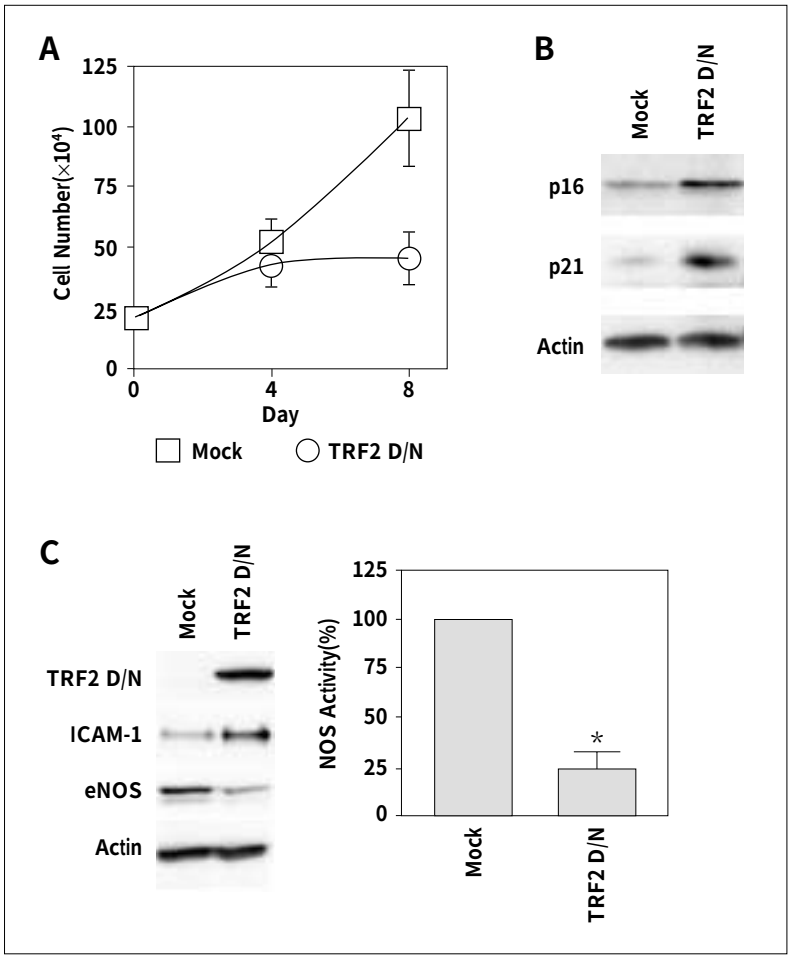

Figure 2 A, Cell growth after infection with TRF2 D/N. Human endothelial cell populations infected with TRF2 D/N or Mock were purified with 1μg/ml puromycin for 3 days and 25×10^4 cells were plated at Day 0. Cell number was then counted at indicated time points (n=4). B, Western blot analysis for CDK inhibitors. The expression levels of p21 and p16 were significantly increased in TRF2 D/N-infected cell populations at Day 4. C, Western blot analysis of ICAM-1, eNOS and TRF2D/N and NOS activity in HAECs. The NOS activity in HAECs infected with LPCX is set at 100% and compared to that in HAECs infected with TRF2 D/N (right graph, n=4, *p<0.001, paired-*t* test).

Vascular cell senescence promotes endothelial dysfunction

Telomere ends form large duplex loops, and telomeric protein TRF2 is essential for their formation. Inhibition of TRF2 has been reported to induce either cellular senescence or apoptosis in various cells.[7, 8] To investigate the effects of telomere malfunction on endothelial functions, we introduced a dominant-negative form of TRF2 lacking both the Myb DNA binding domain and the NH_2-terminal basic domain (TRF2 D/N)[7] into human aortic endothelial cells (HAECs, BioWhittaker) by retroviral infection and examined intercellular adhesion molecule (ICAM)-1 expression and NOS activity. Introduction of TRF2D/N induced a growth arrest with phenotypic characteristics of cellular senescence such as enlarged cell shapes, induction of cyclin-dependent kinase (CDK) inhibitors and increased senescence-associated β-gal activity whereas no evidence for senes-

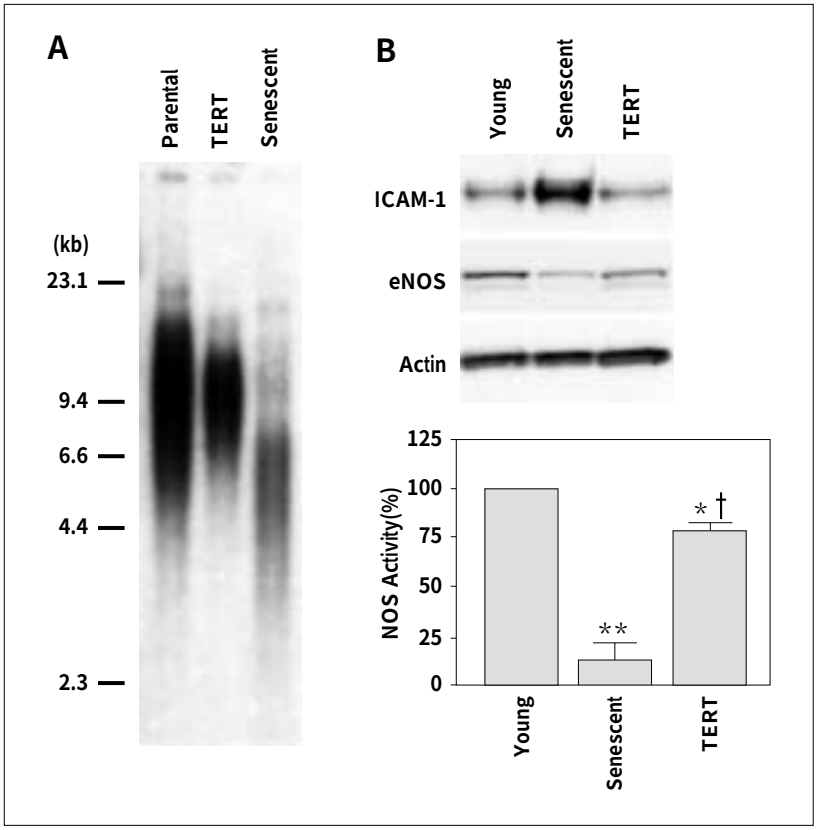

Figure 3 A, Telomere length of HAECs infected with TERT and Mock. Telomere length was stabilized in TERT-infected cells, whereas progressive telomere shortening was observed in Mock-infected cells undergoing senescence. B, Western blot analysis of ICAM-1 and eNOS, and NOS activity in parental young HAECs (20PD), Mock-infected senescent HAECs (49PD) and TERT-infected HAECs (49PD). The NOS activity in parental HAECs is set at 100% and compared to that in senescent HAECs and HAECs infected with TERT (right graph, n=4, *$p<0.05$ vs. parental, **$p<0.001$ vs. parental, †$p<0.01$ vs. senescent, ANOVA). Similar results were observed in three independent Western blot analyses.

cence was seen in the mock-infected cells (**Figs. 2A** and **B**). No apparent apoptotic response was observed after infection with TRF2D/N. The expression of ICAM-1 was significantly increased and the activity of NOS as well as the levels of eNOS protein was reduced after infection with TRF2D/N (**Fig. 2C**). Similar functional changes were observed in endothelial cell populations undergoing replicative senescence by ～50 population doublings (PDs)(**Fig. 3B**), implicating that vascular cell senescence contributes to endothelial dysfunction.

Telomerase protects against endothelial dysfunction associated with senescence

Finally, we examined whether telomerase prevents endothelial dysfunction associated with cellular senescence. In contrast to progressive telomere shortening in mock-infected cells, telomere length was preserved in TERT-infected cells (**Fig. 3A**). Moreover, introduction of TERT significantly extended life span of HAECs, whereas mock-infected cells underwent senescence by ～50 PDs. Significantly decreased levels of ICAM-1 and increased levels of eNOS and NOS activity were detected in TERT-infected cells as compared to those in senescent cells (Fig. 3B), indicating that TERT conferred a protection against endothelial dysfunction associated with replicative senescence.

Discussion

Endothelial dysfunction is considered to be a key event in the evolution of atherosclerotic plaques. The morphological features of endothelial cells during the development of atherosclerosis have been studied extensively.[9] These studies have reported that the large endothelial cells that resemble senescent cells *in vitro* were frequently found on the plaque surface, implicating that vascular cell senescence might occur *in vivo*. We here demonstrated that vascular endothelial cells in

human atherosclerotic lesions exhibited high levels of senescence-associated β-gal activity, as previously reported in human dermal fibroblasts in aging skin *in vivo*.[6] Consistent with our findings, the rate of telomere loss was reported to be greater in the intimal cells of iliac arteries compared to that of the internal mammary arteries, a region of the arterial tree subject to less hemodynamic stress.[3] Thus, it is likely that increased rate of cell turnover in the region of disturbed flow accelerates telomere loss and endothelial senescence, which contributes to endothelial dysfunction as we observed *in vitro*.

Alterations associated with aging in the blood vessels include a decrease in compliance and an increase in vascular inflammatory response that promote atherogenesis. It has been suggested that these alterations are attributed to age-associated functional changes in vascular cells. Endothelial-dependent vasodilatation is impaired with age because of a decrease in endothelial production of nitric oxide whereas adhesion molecules and pro-inflammatory cytokines are increased in endothelial cells, contributing to vascular inflammation.[10] In this study, similar functional changes were observed *in vitro* in aged vascular cells that undergo cellular senescence. Combined with the evidence that endothelial cells with senescence-associated phenotypes exist in human atherosclerotic lesions, it is conceivable that functional changes in senescent endothelial cells *in vivo* may play an important role in the pathophysiology of age-associated vascular disorders.

Several factors, such as oxidative stress and DNA damages have been shown to cause cellular senescence *in vitro*. In this study, endothelial cells became senescent and their functions were altered by inhibition of TRF2 alone, suggesting that telomere function is necessary for endothelial function. This idea is further supported by the evidence that introduction of TERT prevented impaired endothelial function with replicative senescence. However, this prevention appears incomplete, inasmuch as TERT-infected cells exhibited significantly lower activity of NOS as compared to that of parental young cell populations (**Fig. 3C**). Moreover, we failed to establish immortalized endothelial cells by introduction of TERT in contrast to successful immortalization of VSMC as previously reported[2] (T. Minamino unpublished data). Thus, additional activities given by anti-senescent genes may be required to maintain functional integrity as well as long-term cell viability of endothelial cells.

In summary, we showed endothelial cells with senescence-associated phenotypes in human atherosclerotic lesions. Our results imply a crucial role of telomere function in the vasculature and may provide insights into a novel treatment of anti-senescence to prevent atherosclerosis.

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