Endothelial Cell Senescence in Human Atherosclerosis
Role of Telomere in Endothelial Dysfunction

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Background—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 in vivo has been observed in the regions susceptible to atherosclerosis, implying contributions to atherogenesis. However, whether senescent vascular cells are present in the vasculature and contribute to the pathogenesis of atherosclerosis remains unclear.

Methods and Results—Senescence-associated β-galactosidase (β-gal) activity was examined in the coronary arteries and the internal mammary arteries retrieved from autopsied individuals who had had ischemic heart diseases. Strong β-gal stainings were observed in atherosclerotic lesions of the coronary arteries but not in the internal mammary arteries. An immunohistochemical analysis using anti-factor VIII antibody 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 catalytic component significantly extended the life span and inhibited the functional alterations associated with senescence in HAECs.

Conclusions—Vascular endothelial cells with senescence-associated phenotypes are present in human atherosclerotic lesions, and endothelial cell senescence induced by telomere shortening may contribute to atherogenesis. (Circulation. 2002;105:1541-1544.)

Key Words: aging ■ atherosclerosis ■ endothelium

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. We have demonstrated previously that the 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. Progressive telomere shortening in human arteries has been observed in the regions susceptible to atherosclerosis. Moreover, telomere length has been reported to inversely correlate with pulse pressure and atherosclerotic grade in human.

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.

Methods

Tissue Specimens
Human coronary arteries and internal mammary arteries were obtained from 4 autopsied individuals who had ischemic heart diseases.

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The autopsy tissues were obtained within 12 hours after death and were subjected to β-galactosidase (β-gal) staining.

**Histology**

Senescence-associated β-gal activity was examined in the tissues as described previously. Briefly, the samples were incubated for 24 hours at 37°C in freshly prepared β-gal staining solutions containing 1 mg/mL 5-bromo-4-chloro-3-indolyl β-d-galactopyranoside (X-gal), 5 mmol/L potassium ferrocyanide, 5 mmol/L potassium fericyanide, 150 mmol/L NaCl, 2 mmol/L MgCl₂, 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. The frozen sections (6 µm) were subjected to immunohistochemistry.

**Retroviral Infection**

The expression vector of a dominant-negative form of human TRF2 with FLAG tag (TRF2D/N), pTetFLAGhTRF2ΔΔ, was the 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.

**Western Blot**

Western blot analysis was performed as described.

**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 ANOVA 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 (Figure 1A). The cross-sections of arteries stained with β-gal indicated that β-gal–positive cells were mostly located on the luminal surface (Figure 1B, left). The high-magnification photograph in Figure 1B demonstrated granular blue stainings in the cytoplasm of the cells on the luminal surface. We also found the colocalization of similar granular stainings with an immunoreactivity for α-smooth muscle actin (Data Supplement), indicating that the blue stainings originated from cells, not from an 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 (Figure 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 the round shape of endothelial cells in nonatherosclerotic lesions. These were predominately localized on the surface of atherosclerotic plaques, suggesting that vascular endothelial cell senescence may be involved in atherogenesis.

**Vascular Cell Senescence Promotes Endothelial Dysfunction**

Telomeres end 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. 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 NH₂-terminal basic domain (TRF2 D/N) into human aortic endothelial cells (HAECs, BioWhittaker) by retroviral infection and examined intercellular adhesion molecule-1 (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 senescence was seen in the mock-infected cells (Figure 2A and Data Supplement). No apparent apoptotic response was observed after infection with
Telomerase Protects Against Endothelial Dysfunction Associated With Senescence

Finally, we examined whether telomerase prevents endothelial dysfunction associated with cellular senescence. Introduction of TERT significantly extended life span of HAECs, whereas mock-infected cells underwent senescence by \( \approx 50 \) PDs (Figure 2A). Significantly decreased levels of ICAM-1 and increased levels of eNOS and NOS activity were detected in TERT-infected cells as compared with those in senescent cells (Figure 2C), 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. These studies have reported that the large endothelial cells that resemble senescent cells in vitro were frequently found on the plaque surface, implying that vascular cell senescence might occur in vivo. In this study, we demonstrated that vascular endothelial cells in human atherosclerotic lesions exhibited high levels of senescence-associated \( \beta\)-gal activity, as previously reported in human dermal fibroblasts in aging skin in vivo. Consistent with our findings, the rate of telomere loss was reported to be greater in the intimal cells of iliac arteries than in the internal mammary arteries, a region of the arterial tree subject to less hemodynamic stress. Thus, it is likely that an 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, both of which 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. 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 damage, 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 the introduction of TERT prevented impaired endothelial function with replicative senescence. However, this prevention appears incomplete, inasmuch as TERT-infected cells exhibited significantly lower NOS activity than did parental young cell populations (Figure 2C). Moreover, we failed to establish immortalized endothelial cells by introduction of TERT, in contrast to the successful immortalization of vascular smooth muscle cells as previously reported (T. Minamino, unpublished data, August 1, 2001). Thus, additional activities given by antisenescent genes may be required to maintain functional integrity as well as long-term cell viability of endothelial cells.
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 antisenescence to prevent atherosclerosis.

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