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Role of telomeres in vascular senescence

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1. ABSTRACT

Telomeres are DNA regions composed of TTAGGG repeats that are located at the ends of chromosomes. Specific proteins associate with the telomeres and form non-nucleosomal DNA-protein complexes that serve as protective caps for the chromosome ends. There is accumulating evidence that progressive telomere shortening is closely related to cardiovascular disease. For example, vascular cell senescence has been reported to occur in human atherosclerotic lesions and this change is associated with telomere shortening. Impairment of telomere integrity causes vascular dysfunction, which is prevented by the activation of telomerase. Mice with short telomeres develop hypertension and exhibit impaired neovascularization. Short telomeres have also been reported in the leukocytes of patients with cardiovascular disease or various cardiovascular risk factors. Although it remains unclear whether short telomeres directly cause cardiovascular disease, manipulation of telomere function is potentially an attractive strategy for the treatment of vascular senescence.

2. INTRODUCTION

Epidemiological studies have shown that age is the dominant risk factor for atherosclerotic cardiovascular disease (1, 2). The incidence and the prevalence of atherothrombotic diseases, including coronary heart disease and stroke, both increase with advancing age (1, 2). However, the molecular mechanisms underlying the increased risk of such diseases due to aging remain unclear. For example, arterial stiffness increases with age due to structural changes of the walls of arteries as well as endothelial dysfunction, but convincing explanations at the molecular level for these age-associated alterations of vascular structure and function have not yet been discovered.

Cellular senescence occurs due to the limited ability of normal cells to divide *in vitro* and is accompanied by specific changes of cell morphology, gene expression, and function. The occurrence of such changes has been suggested to play a role in human aging (3). This hypothesis (the cellular hypothesis of aging) was first described by Hayflick in the 1960s and is supported by the

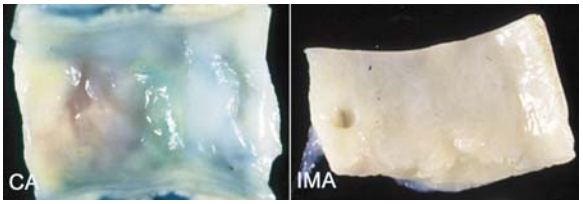

Figure 1. SA β-gal-positive vascular cells in human atheroma. Photographs of the luminal surface of human coronary artery (CA, left) and internal mammary artery (IMA, right) stained with β-gal staining. Senescent-associated β-gal activity was observed in human coronary arteries but not in internal mammary arteries. Adapted from ref (14) with permission.

finding that primary cultured cells from patients with premature aging syndromes, such as Werner syndrome and Bloom syndrome, have a shorter lifespan than cells from age-matched healthy persons (4, 5). Until recently, however, little attention has been paid to the potential impact of vascular cell senescence on age-related vascular disorders.

Over the past few decades, significant progress has been made in our understanding of the role of telomeres in cellular senescence, enabling us to reassess the cellular hypothesis of aging with regard to human vascular disorders. For example, it may be possible that cellular senescence contributes to age-associated vascular diseases or that telomere shortening promotes vascular aging. In this review, we will describe recent evidence that supports the cellular hypothesis of aging in relation to the vasculature and discuss the potential involvement of cellular senescence induced by telomere shortening in the pathogenesis of human vascular disorders.

3. VASCULAR CELL SENESCENCE *IN VIVO*

Vascular cells have a finite lifespan *in vitro* and eventually enter a state of irreversible growth arrest called cellular senescence. Flattening and enlargement of vascular cells are known as morphological characteristics of senescence (6). Expression of negative regulators of cell cycle (such as p53 and p16) increases with cell division and thereby promotes growth arrest (7). Primary cultured cells that undergo senescence *in vitro* also show increased expression of β-galactosidase (β-gal) activity at pH 6, which is distinguishable from endogenous lysosomal β-gal activity that can be detected at pH 4. The activity at pH 6 is known as senescence-associated β-gal (SA β-gal) activity, and it shows a correlation with the aging of cells and thus is regarded as a biomarker for cellular senescence (8). The *in vitro* growth of vascular cells obtained from human atherosclerotic plaques is impaired, and such cells develop senescence earlier than cells harvested from normal vessels (9, 10). The histology of human atherosclerotic lesions has been extensively studied, and it has been demonstrated that both vascular endothelial cells and vascular smooth muscle cells (VSMCs) exhibit the morphological features of cellular senescence (11, 12). These findings suggest the occurrence of vascular cell senescence *in vivo*.

In fact, this hypothesis has been confirmed by *in vivo* cytochemical analysis of SA β-gal activity. Fenton *et al.* detected SA β-gal-positive vascular cells in damaged rabbit carotid arteries (13). After repeated endothelial denudation, accumulation of SA β-gal-positive cells was markedly enhanced. We have previously demonstrated SA β-gal-positive vascular cells in atherosclerotic plaques obtained from the coronary arteries of patients with ischemic heart disease (14). These SA β-gal-positive cells were predominately localized on the luminal surface of the atherosclerotic plaques and were identified as endothelial cells, while such cells were not observed in the internal mammary arteries of the same patients where atherosclerotic changes were minimal (Figure 1). In advanced plaques, however, SA β-gal-positive VSMCs were detected in the intima and not in the media (15). This may have been due to extensive cell replication in the lesions, as is observed in arteries subjected to double denudation. SA β-gal-positive cells in human atheroma exhibit increased expression of p53 and p16 (other markers of cellular senescence), which is further evidence in favor of *in vivo* senescence. These cells also show various functional abnormalities such as decreased expression of endothelial NO synthase (eNOS) and increased expression of pro-inflammatory molecules (15). Thus, cellular senescence may contribute to the pathogenesis of vascular aging in humans.

4. MECHANISMS OF CELLULAR SENESCENCE

4.1 Telomeres and telomerase

One widely discussed hypothesis of cellular senescence is the telomere hypothesis (16). Telomeres are non-nucleosomal DNA-protein complexes located at the ends of chromosomes that serve as protective caps and act as a substrate for specialized replication mechanisms. As a consequence of semi-conservative DNA replication, the extreme terminals of the chromosomes are not duplicated completely, resulting in shortening of the telomeres with each cell division. A critical reduction of telomere length is thought to trigger the onset of cellular senescence. Thus, telomere shortening has been proposed to act as a mitotic clock that prevents the unlimited proliferation of human somatic cells.

Telomerase is a ribonucleoprotein that adds telomeres to the ends of chromosomes using its RNA moiety as a template. Early studies detected telomerase activity in cancer cells and stem cells, but not in normal somatic cells, suggesting that telomerase might be essential for tumor growth and the self-renewal potential of stem cells (17, 18). However, evidence has since emerged that telomerase also regulates the proliferation of normal somatic cells by lengthening of telomeres or by telomere length-independent mechanisms (19-21). Human endothelial cells and VSMCs express telomerase activity, which is markedly enhanced by mitogenic stimuli (22). This activity declines with cellular aging *in vitro* due to a decrease of TERT expression, leading to telomere

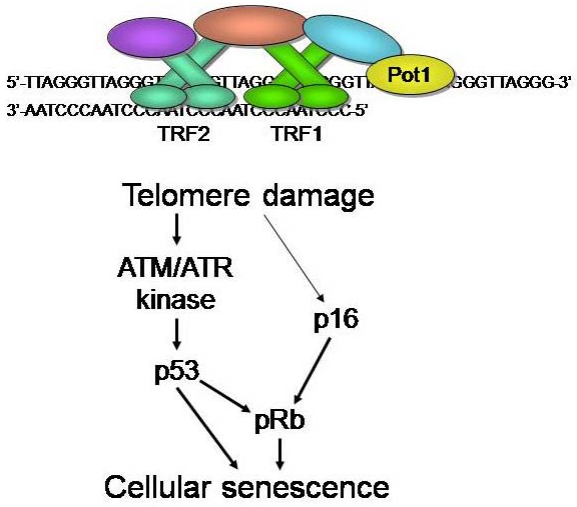

Figure 2. The shelterin complex and the signaling pathways in response to telomere damage. Upper panel depicts the six subunits of shelterin. Telomere damage activates the ATM/ATR kinases, which leads to a p53-dependent cell cycle arrest. The p16/pRb pathway also mediates telomere-dependent senescence.

shortening and the occurrence of cellular senescence (23, 24). The introduction of telomerase extends the lifespan of both endothelial cells and VSMC (23, 25, 26), suggesting a critical role of telomeres and telomerase in vascular cell senescence.

Oxidative stress has been suggested to have a role in human aging as well as cellular senescence (27). Chronic oxidative stress caused by exposure to chemical oxidants induces telomere shortening and accelerates the onset of senescence (28), as well as senescence-associated death (29), in human endothelial cells. Homocysteine is one of the known risk factors for atherosclerosis, and it has been reported to induce telomere shortening and accelerate endothelial cell senescence (30). Oxidized low-density lipoprotein has been reported to inactivate telomerase by inhibiting the phosphoinositol 3-kinase/Akt pathway in vascular endothelial cells (31), thus inducing premature senescence (32). Conversely, suppression of oxidative stress or hypoxia preserves telomere length and extends the lifespan of cells, at least partly through an increase of telomerase activity (23, 33, 34). NO also activates telomerase and delays the onset of endothelial cell senescence. It is possible that NO scavenges intracellular radicals and thus decreases oxidative stress, resulting in the activation of telomerase (35).

4.2 Factors acting downstream of telomere dysfunction

Although diverse stimuli can induce cellular senescence, they appear to converge mainly on either or both of two pathways that establish and maintain the process of senescence. These pathways are regulated by the tumor suppressor proteins p53 and pRb (36, 37). Both proteins are transcriptional regulators, and each lies at the center of signaling pathways responsible for cell cycle regulation, DNA repair, and cell death, which include a number of upstream regulators and downstream effectors (38). p53 is a crucial mediator of the cellular response to DNA damage and it induces the cyclin-dependent inhibitor p21 (39). Dysfunctional telomeres resemble damaged DNA and thus trigger a p53-dependent response (40). Recent studies have demonstrated that nuclear foci containing markers for double-stranded DNA breaks form in cells with critically short or dysfunctional telomeres (41, 42), and it has been shown that such foci are increased in fibroblasts of aging primates (43).

It has been postulated that telomeres form large duplex loops (called t-loops) with telomeric proteins, which protect the ends of chromosomes. Mammalian telomeres have been reported to associate with the shelterin complex (44). This complex consists of three subunits that directly recognize TTAGGG repeats, which are known as protection of telomeres 1 (Pot1) (45), telomeric repeat binding factor 1 (TRF1), and TRF2 (46), and these subunits are interconnected by three additional shelterin proteins (Figure 2). Pot-1 has been identified as a telomeric protein that binds to the tips of telomeres and its deletion elicits a DNA damage response at the sites of telomeres, resulting in p53-dependent senescence (47, 48). TRF1 was originally reported to be mainly involved in the regulation of telomere length (49), whereas TRF2 was implicated in protecting chromosome ends (50). However, recent reports have shown that both proteins play a crucial role in regulating telomere length as well as in protecting chromosome ends (51, 52). Although the DNA damage response activated by inhibition of shelterin is thought to be p53-dependent, there is some evidence that the p16/pRb pathway is also activated by telomere dysfunction (53). In addition to shelterin, telomeres interact with a number of other factors that could influence telomere integrity, including Ku70, Ku86, DNA-dependent protein kinase, poly (ADP-ribose) polymerase (PARP), the RecQ-like helicases, and exision repair cross complementing 1 (ERCC1) (54). These factors are involved in the processes of DNA recombination and repair, and thus could contribute to telomere damage as well as to telomere stability.

4.3 Telomere-independent signaling pathways

It is now apparent that cellular senescence can also be induced by various stresses independently of the replicative age of a cell (36, 55). Cells undergo senescence when DNA damage occurs that is irreparable or threatens to overwhelm the DNA repair machinery (56). Supraphysiological mitogenic signals that result from the overexpression of oncogenes also elicit the senescence of many normal cells (36). Finally, cells can undergo senescence in response to epigenetic changes of chromatin organization that may alter the expression of proto-oncogenes or tumor suppressor genes (57). Thus, it is possible that atherogenic stimuli may increase cell turnover at sites of atherosclerosis, thereby promoting telomere shortening, and possibly also activating certain proliferative signals that induce senescence independently of telomere shortening. Consistent with this notion, we have demonstrated that atherogenic stimuli like angiotensin II and insulin promote vascular cell senescence, thereby promoting vascular dysfunction (58-60). Moreover, oxidative stress and DNA damage may promote vascular

cell senescence and thus further potentiate atherogenesis (58).

5. PROGRESSIVE TELOMERE SHORTENING IN AGE-ASSOCIATED VASCULAR DISEASES

There is also evidence that telomere shortening occurs in human vessels and that this process may be related to age-associated vascular diseases. The telomere length of endothelial cells from the abdominal aorta and iliac arteries shows a strong inverse correlation with age (25, 61). Importantly, telomere shortening occurs more rapidly in endothelial cells from the iliac arteries compared with those from the internal mammary arteries (25). Thus, greater hemodynamic stress may enhance the rate of endothelial cell turnover in the iliac arteries compared with that in vessels subjected to less hemodynamic stress. Telomere shortening is also more advanced in coronary artery endothelial cells obtained from patients with coronary heart disease compared with cells from healthy subjects (62). Furthermore, it has also been reported that fibrous cap VSMCs have markedly shorter telomeres than normal medial VSMCs (63).

The telomere length of white blood cells from healthy subjects shows an inverse correlation with their pulse pressure that is independent of chronological age, at least in men (64). The telomeres of white blood cells from patients with severe coronary artery disease are significantly shorter than those of cells from healthy controls, which might reflect the accelerated biological aging of various tissues (including the coronary arteries) (65). In fact, the risk of myocardial infarction is increased by ~3-fold in subjects with short telomeres (66), and this increased risk associated with shorter telomeres can be ameliorated by statin treatment (67). Short telomeres are associated with an increase of carotid atherosclerosis in hypertensive subjects (68). Degenerative aortic valve stenosis is also correlated with telomere shortening in the elderly, and this correlation is independent of coronary heart disease (69). Furthermore, short telomeres are found in patients with vascular dementia (70). It has been reported that various risk factors for cardiovascular disease, such as obesity, smoking, psychological stress, insulin resistance, hypertension, and diabetes, are associated with reduced telomerase activity or telomere shortening in white blood cells (71-76). Cawthon *et al.* (77) examined the white blood cell telomere length in 143 normal unrelated subjects over the age of 60 years and found that those with shorter telomeres had worse survival, which was attributable to a 3.18-fold higher mortality rate from heart disease and an 8.54-fold higher mortality rate from infections. In contrast, two other recent studies found no association between telomere length and mortality in the elderly (78, 79). It also remains unclear whether the length of white blood cell telomeres is related to that of vascular cells.

6. CELLULAR SENESCENCE AND VASCULAR DYSFUNCTION

Age-associated changes of the blood vessels include a decrease in compliance and an increase of the inflammatory response that promote atherogenesis (80). It has also been reported that angiogenesis becomes impaired with advancing age (81, 82), and that aging decreases the antithrombogenic properties of the endothelium (83). A number of studies have shown that many of the changes detected in senescent vascular cells are consistent with the known changes that occur in age-related vascular diseases, suggesting a critical role of cellular senescence in vascular pathophysiology. For example, NO production and eNOS activity are reduced in senescent human endothelial cells (84), and the induction of NO production by shear stress is also decreased in senescent endothelial cells (85). The decline of eNOS activity in senescent endothelial cells is attributable to a decrease of eNOS protein expression, as well as a decrease of eNOS phosphorylation mediated by Akt (86). Prostacyclin production shows a significant decrease with the *in vitro* aging of endothelial cells (87), while senescent endothelial cells display upregulation of plasminogen activator inhibitor-1 (88). These alterations are probably involved in the impairment of endothelium-dependent vasodilation and also increase the risk of thrombogenesis in humans with atherosclerosis.

Interactions between monocytes and endothelial cells are enhanced by endothelial cell senescence (89), thereby promoting atherogenesis. This change appears to be mediated by the upregulation of adhesion molecules and pro-inflammatory cytokines, as well as decreased production of NO by senescent endothelial cells (14, 85). It has been reported that the ability of senescent endothelial cells to form capillary structures *in vitro* is reduced (26). Bone marrow-derived circulating endothelial progenitor cells (EPCs) are known to participate in postnatal neovascularization and vascular repair (90, 91). The *in vitro* growth and function of bone marrow-derived EPCs from patients with coronary artery disease are impaired and these changes are correlated with coronary risk factors including age (92, 93). Thus, aging may promote the senescence of EPCs as well as endothelial cells, resulting in decreased angiogenesis and vascular healing.

7. TELOMERE IMPAIRMENT AND VASCULAR DYSFUNCTION

In most of the previous studies mentioned above, the phenotypic changes associated with senescence were studied in vascular cell populations undergoing replicative senescence, and thus were suggested to be related to telomere-dependent vascular dysfunction. However, it remains unclear whether the phenotypic changes of senescent vascular cells actually result from telomere dysfunction. Inhibition of TRF2 has been shown to induce either senescence or apoptosis of various cells by destroying telomere loops (50, 94). It has also been demonstrated that the introduction of a dominant-negative form of TRF2 into human endothelial cells induces growth arrest along with phenotypic characteristics of cellular senescence (14). Telomere dysfunction significantly increases ICAM-1 expression and reduces eNOS activity, suggesting a causal link between telomeres and vascular dysfunction associated with cellular senescence.

Telomerase-deficient mice show a normal phenotype in the first generation, presumably because these animals have very long telomeres (19, 95). However, their telomeres become shorter with successive generations, and they become infertile by the sixth generation due to impairment of the reproductive system. In some respects, the later-generation mice mimic changes associated with aging. They have a shortened lifespan and a reduced capacity to respond to stresses such as wounds and hematopoietic ablation (96). Neovascularization is also impaired in the later generations of telomerase-deficient mice (97), and such decreased vessel formation may be attributable to the impaired function and replication of endothelial cells induced by telomere shortening. In a mouse model of atherosclerosis, telomere shortening has been shown to decrease the area of atherosclerotic lesions, presumably due to reduced macrophage proliferation (98). However, telomerase-deficient mice develop atherosclerotic plaques with a thin fibrous cap, suggesting that shortening of the telomeres of vascular cells may induce plaque rupture in patients with atherosclerosis. Mice lacking telomerase activity develop hypertension in the first and third generations as a result of an increased plasma endothelin-1 level due to the overexpression of endothelin-converting enzyme (99).

8. RESTORATION OF VASCULAR DYSFUNCTION BY TELOMERASE

Introduction of TERT prevents endothelial dysfunction associated with senescence such as decreased eNOS activity and increased monocyte binding to endothelial cells (14, 85). Immortalized human endothelial cells (TERT-ECs) have been established by introduction of TERT (26). TERT-ECs appear to retain their endothelial cell characteristics, including various cell surface markers. When cultured in Matrigel, these cells form capillary-like structures in response to extracellular matrix signals as efficiently as early-passage endothelial cells, whereas senescent or transformed endothelial cells do not. In addition, TERT-ECs are more resistant to the induction of apoptosis than presenescent endothelial cells. They maintain normal growth control and do not exhibit a transformed phenotype. TERT-ECs are also functional *in vivo*, as demonstrated in a Matrigel implantation mouse model (100). In this model, the vessel density achieved with primary cultured human endothelial cells decreases with time after implantation, while durable vessels persist after implanting TERT-ECs, indicating that telomerase activity is important for maintenance of the microvasculature. It has also been reported that TERT acts as an angiogenic factor and is a downstream effector after VEGF signaling (101).

Introduction of TERT into EPCs has been shown to extend the lifespan of these cells and to increase the efficacy of vasculogenesis *in vivo* (102). An obstacle to the effective engineering of human tissues is the limited replicative capacity of adult somatic cells. However, it has been demonstrated that telomerase expression in vascular cells isolated from elderly patients allows the successful culture of engineered autologous blood vessels (103).

9. PERSPECTIVES

The molecular biology of vascular aging has only been studied recently and this field is still in its infancy. However, the recent demonstration that vascular cell senescence occurs *in vivo* along with vascular aging has suggested a pathological role of this process in human atherosclerosis. In addition, the finding that introduction of telomerase immortalizes vascular cells and maintains a juvenile phenotype *in vitro* and *in vivo* may be an important for the future research in the fields of stem cell biology as well as tissue engineering. Future challenges are to determine whether vascular cell senescence actually promotes vascular aging *in vivo* and whether the inhibition of cellular senescence is protective against age-associated vascular diseases.

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Abbreviations: β-gal : β-galactosidase, SA: senescence-associated, VSMC: vascular smooth muscle cells, NO:

nitric oxide, eNOS: endothelial NO synthase, TRF: telomeric repeat binding factor, EPC: endothelial progenitor cell

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