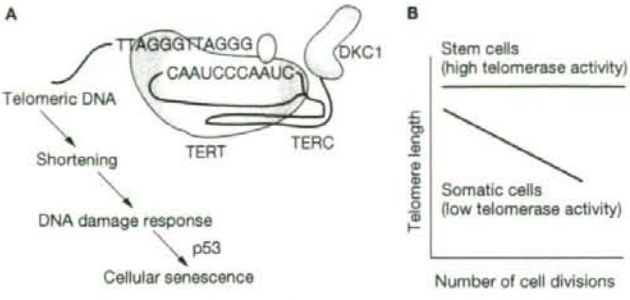


Figure 1 Telomeres and telomerase. (**A**) Telomerase consists of a RNA component, a catalytic component and cofactors including DKC1. The RNA moiety serves as a template for the synthesis of new telomeric repeats by the catalytic component. Critically short telomeres resemble damaged DNA and thus trigger cellular senescence via a p53-dependent pathway. (**B**) Stem cells have high telomerase activity and maintain telomere length, whereas most somatic cells including vascular cells show progressive telomere shortening due to low telomerase activity. Abbreviations: DKC1, dyskeratosis congenita 1, dyskerin; TERC, telomerase RNA component; TERT, telomerase reverse transcriptase.

contributes to age-associated alterations of cardiovascular structure and function.[5,6] In this Review we discuss the potential impact of cellular senescence of vascular and stem cells on age-related cardiovascular disease and examine experimental changes in relation to premature aging syndromes.

CELLULAR SENESCENCE AND VASCULAR AGING

Replicative senescence was originally defined by the finite replicative life span of human somatic cells in culture. Senescent cells enter irreversible growth arrest, exhibit a flattened and enlarged shape, and express a set of genes—including negative regulators of the cell cycle such as p53 and p16—that differs from those normally expressed. The growth potential of cultured cells correlates well with the mean maximum life span of the species from which the cells are derived; therefore, phenotypic changes associated with senescence have been suggested to be involved in human aging. This hypothesis of cellular aging is supported by the finding that primary cells from patients with progeroid syndromes have a shorter life span than cultured cells from age-matched healthy persons.[7] A number of studies have shown that many of the changes seen in senescent vascular cells, such as decreased production of nitric oxide, are consistent with the aforementioned cellular changes seen in patients with age-related cardiovascular disease, suggesting that cellular senescence has a role in the aging of the cardiovascular system.

In vivo evidence of vascular cell senescence

Human atherosclerotic lesions have been studied extensively, and within the plaques studied both endothelial cells and VSMCs have been found that exhibit the morphological features of cellular senescence.[8,9] Vascular cells that are positive for senescence-associated β-galactosidase (SAβ-gal) activity, a biomarker of senescence, have been found in atherosclerotic plaques obtained from the coronary arteries of patients with ischemic heart disease.[10] These SAβ-gal-positive cells are predominately localized on the luminal surface of the plaque and have been identified as endothelial cells. Interestingly SAβ-gal-positive cells have not been observed in the internal mammary arteries of the same patients, where atherosclerotic changes are minimal.[10] In advanced plaques, however, SAβ-gal-positive VSMCs are only detected in the intima and not in the media,[11] possibly the result of extensive replication in these lesions. SAβ-gal-positive VSMCs cells from human atheromas show increased expression of p53 and p16—both markers of cellular senescence.[11] These cells also exhibit various functional abnormalities, including decreased expression of endothelial nitric oxide synthase and increased expression of proinflammatory molecules.[11] Such findings support the theory that cellular senescence occurs *in vivo* and contributes to the pathogenesis of human atherosclerosis.

Telomere shortening in aged arteries

Telomeres are non-nucleosomal DNA–protein complexes located at the ends of chromosomes and serve as protective caps and act as the substrate for specialized replication mechanisms. As a consequence of semiconservative DNA replication, the extreme terminals of chromosomes are not duplicated completely, causing successive shortening of the telomeres with each round of cell division. Telomerase is an enzyme that adds telomeres to the ends of chromosomes.[12] This enzyme consists of a RNA component, a catalytic component, and various cofactors including dyskeratosis congenita 1, dyskerin. The RNA moiety serves as a template for new telomeric repeats synthesized by the catalytic component (Figure 1A). In contrast with

stem cells, which have a high level of telomerase activity and demonstrate consistent telomere length, most somatic cells including vascular cells show progressive telomere shortening due to low telomerase activity (Figure 1B). Critically short telomeres—those that cannot successfully complete replication—resemble damaged DNA and thus trigger cellular senescence via a p53-dependent pathway (Figure 1A).[13] Studies have demonstrated that nuclear foci containing markers of double-stranded DNA breaks form in cells with critically short or dysfunctional telomeres,[14,15] and that the number of such nuclear foci induced by telomere dysfunction increases in the fibroblasts of aging primates.[16]

Telomere shortening also occurs in human vessels and could be related to the development of atherogenesis. The length of telomeres isolated from endothelial cells of human arteries shows a strong inverse correlation with age.[17,18] Interestingly, telomere shortening over time occurs faster in the endothelial cells of the iliac arteries than in those of the internal mammary arteries.[17] Thus, a high level of hemodynamic stress, such as that seen in the iliac arteries, could increase endothelial cell turnover to levels higher than those seen in vessels that are subject to less stress. Of note, telomeres are shorter in coronary artery endothelial cells from patients with coronary heart disease than in the same cells from healthy individuals.[19] Voghel *et al.* demonstrated that endothelial telomere length was shorter in patients with a long history of risk factors for cardiovascular disease than in those who had been at risk for less time.[20] These findings suggest that cardiovascular risk factors override the effects of chronological aging on endothelial cell turnover by accelerating stress-induced damage. Identification of factors that accelerate the attrition of endothelial telomere length will facilitate the development of targeted treatments for human atherosclerosis.

Role of telomeres in vascular senescence

Investigations have shown that disturbance of telomere integrity can lead to endothelial dysfunction *in vitro*.[10] Human endothelial cells and VSMCs demonstrate telomerase activity, which is markedly increased by mitogenic stimuli;[21] however, as the cells age this activity declines because of decreased expression of the catalytic component of telomerase, leading to telomere shortening and cellular senescence.[22] The introduction of telomerase into human endothelial cells *in vitro* prevents progression to senescence-associated endothelial dysfunction, including the decrease in endothelial nitric oxide synthase activity and increase in monocyte adhesion to endothelial cells.[10,23] Introduction of telomerase has been used as a method to produce immortalized human endothelial cells, which seem to retain endothelial cell characteristics including various cell-surface markers.[24] When cultured in Matrigel® (BD Biosciences, San Jose, CA), these immortalized cells form capillary-like structures as efficiently as healthy, young endothelial cells.[25]

First-generation, telomerase-deficient mice have a normal phenotype, presumably because mice possess longer telomeres than humans.[26,27] With successive generations, however, the telomeres become shorter. By the sixth generation these mice become infertile due to impairment of their reproductive system.[27] Some of the abnormalities seen in later generations of telomerase-deficient mice mimic age-associated changes. For example, these animals have a shortened life span and a reduced capacity to respond to stresses such as wounds and hematopoietic ablation.[28] Later generations of telomerase-deficient mice also have impaired neovascularization,[29] which could be attributable to impairment of the function and replication of vascular endothelial cells by telomere shortening. In a mouse model of atherosclerosis, Poch *et al.* showed that telomere shortening decreased the size of atherosclerotic lesions, presumably due to reduced proliferation of macrophages.[30] Telomerase-deficient mice, however, develop atherosclerotic plaques with thin fibrous caps, indicating that shortening of vascular-cell telomeres could lead to plaque rupture in human atherosclerosis. First and third generation mice lacking telomerase develop hypertension as a result of an increase in levels of plasma endothelin 1 caused by overexpression of endothelin-converting enzyme. This upregulation is mediated by increased levels of reactive oxygen species and involves an activator-protein-1-dependent mechanism.[31]

Stress-induced premature senescence

In response to various stress signals, cells can develop a phenotype indistinguishable from that of senescent cells at the end of their replicative life span. For example, the constitutive activation of mitogenic stimuli by expression of the *ras* oncogene induces a senescent phenotype

in vascular cells.[11] Cellular senescence triggered by mitogenic stimuli is independent of replicative age, and mitogenic signals influence cell function before the replicative limit of a cell is reached. This form of senescence is apparently telomere-independent and thus termed 'stress-induced premature senescence'. Angiotensin II is a potent mitogen and one of the main effectors of *ras*. Arterial expression of angiotensin II increases with age, and this upregulation is thought to contribute to the pathogenesis of atherosclerosis. Inhibition of angiotensin II activity has been demonstrated to improve cardiovascular-related morbidity and mortality.[32] Angiotensin II has been reported to induce premature senescence of human VSMCs *in vitro* via the p53/p21-dependent pathway.[33] Furthermore, angiotensin II increased the proportion of senescent VSMCs and induced the expression of proinflammatory molecules, as well as p21, in aortic atherosclerotic lesions in a mouse model of atherosclerosis.[33] By knocking out p21 in these mice, the induction of proinflammatory molecules by angiotensin II was markedly reduced, thereby preventing the development of atherosclerosis.

Oxidative stress and DNA damage can induce premature senescence in vascular cells, and it has been theorized that both processes contribute to atherogenesis.[33,34] Evidence indicates that subjecting human endothelial cells to chronic oxidative stress, including exposure to oxidized LDL, enhances telomere shortening and accelerates the onset of senescence.[35] Conversely, treating endothelial cells isolated from patients with severe coronary heart disease with antioxidants can preserve telomere length and extend cell life, unless the oxidative stress-induced damage is severe and therefore irreversible.[36] Oxidative stress targets DNA among other cellular targets, and could induce DNA damage. Many different types of oxidative-stress-related DNA lesions have been described, ranging from base modifications to single-strand and double-strand breaks.[37]

To cope with DNA damage, cells have evolved DNA repair systems. Some strains of mice that lack components of these systems exhibit early onset of changes associated with aging, comparable to those seen in humans.[37] Fibroblasts from these mice also show accelerated senescence compared with normal counterparts.[37] In another set of mouse studies, constitutive activation of p53 caused premature aging characterized by a reduced life span, osteoporosis, organ atrophy and diminished stress tolerance.[38,39] More importantly, phenotypic evidence of cellular senescence has been detected *in vivo* by studies of mice with premature aging disorders.[40] Considered together with the data from studies of telomerase-deficient mice, these results provide *in vivo* evidence that links cellular senescence to aging of the organism in question.

Although there are only a few reports indicating that mice with premature aging are prone to developing atherosclerosis, it is assumed that atherogenic stimuli such as oxidized LDL and angiotensin II increase cell turnover at sites of atherosclerosis and thus promote telomere shortening and possibly trigger oxidative stress-induced DNA damage. It is likely that senescence of vascular cells is triggered by both telomere-dependent and telomere-independent mechanisms, and that both mechanisms are involved in the development of human atherosclerosis.

SENESCENCE OF ENDOTHELIAL PROGENITOR CELLS

Maintenance of a healthy endothelium is essential for blood vessels to function properly and prevents the development of vascular diseases such as atherosclerosis. With increasing age, endothelial integrity becomes progressively impaired. Damaged endothelial cells can be replaced through replication of the surrounding endothelial cells, a process that induces senescence of the surrounding endothelium.

Effect of age on number and function of endothelial progenitor cells

Bone-marrow-derived circulating endothelial progenitor cells (EPCs) have been identified in the peripheral blood in humans[41,42] and have been shown to contribute to both physiological and pathological vascularization in adults.[43] Accumulating evidence suggests that circulating EPCs also have a critical role in vascular repair,[44] although the precise role(s) of EPCs is still controversial.[45] Age-dependent impairment of vascular repair and neovascularization after tissue ischemia might be a consequence of reduced availability and impaired function of EPCs, as well as the limited regenerative capacity of mature endothelial cells (Figure 2).

Consistent with this notion, the number of EPCs in healthy individuals is reported to

reduce with increasing age.[46] Patients with coronary artery disease (CAD) have lower levels of EPCs than healthy controls, and the number of EPCs in patients with CAD also decreases with age, independent of any coronary risk factors.[47] Interestingly, reports suggest that the number of risk factors for cardiovascular disease is significantly correlated with the levels of circulating EPCs. Moreover, *in vitro* migration analysis has demonstrated that EPC function is severely impaired in patients with CAD and that this impairment becomes more serious with increasing age.[47] In patients undergoing CABG surgery, mobilization of EPCs was found to be impaired in older patients compared with younger individuals, suggesting that the responsiveness of EPCs declines with increasing age.[48] Hill *et al.* reported a strong correlation between number of EPCs and Framingham risk score in a population of individuals with no history of cardiovascular disease.[49] Moreover, flow-mediated vasodilation is significantly correlated with number of circulating EPCs.[49]

Transplantation of bone-marrow-derived mononuclear cells (BMCs) into ischemic tissues has been shown to improve perfusion in young mice (8 weeks), but the effect is markedly weaker when BMCs from older animals (18 months) are used.[50] Heeschen *et al.* transplanted BMCs into ischemic hind limbs of nude mice and found that blood flow recovery was significantly impaired in the group treated with BMCs from subjects with CAD compared with those who received BMCs from healthy subjects.[51] Likewise, incorporation of BMCs from subjects with CAD into vascular structures of ischemic limbs was markedly reduced. Consistent with the results of animal studies, recent clinical trials have reported limited benefit of autologous cell therapy in patients with CAD.[52] Conversely, age-associated impairment of cardiac angiogenesis in mice can be reversed by implantation of bone-marrow-derived EPCs from young animals.[53] Moreover, chronic treatment with bone-marrow-derived EPCs from young mice deficient in apolipoprotein E (apoE) prevents the progression of atherosclerosis in apoE-deficient recipients, but the effectiveness of this therapy is reduced when the donor is older.[54] In apoE-deficient recipients, this treatment seems to prevent endothelial senescence and vascular inflammation, as analysis of treated animals shows that they have longer telomeres in vascular cells and lower plasma levels of cytokines. Likewise, systemic transfusion of splenic mononuclear cells can ameliorate endothelial dysfunction in apoE-deficient mice.[55] These results suggest that age-associated deterioration of EPCs contributes to the impairment of vascular repair in elderly subjects, thereby increasing the morbidity caused by cardiovascular disease. This impairment needs to be considered when elderly patients are treated with autologous EPCs.

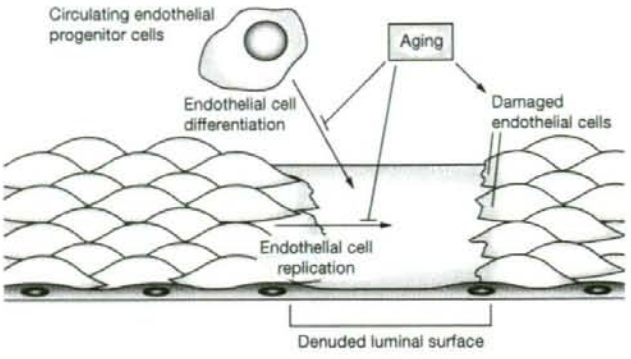


Figure 2 Endothelial repair. Replacement of damaged endothelial cells could occur through replication of surrounding endothelial cells. Bone-marrow-derived circulating endothelial progenitor cells also have a crucial role in endothelial repair. The number drops and the function of these cells become progressively impaired with age, while an age-associated increase of endothelial damage causes exhaustion of the endothelial repair system, thereby inducing cardiovascular aging.

Potential mechanisms of age-associated dysfunction of endothelial progenitor cells

Various factors seem to be involved in age-associated deterioration of EPC number and function. Reports suggest that cardiovascular risk factors such as dyslipidemia, hypertension and diabetes mellitus affect the number and function of circulating EPCs,[6] and that a reduced level of circulating EPCs can predict the occurrence of cardiovascular events.[56] EPC dysfunction could result from accelerated senescence (caused by CAD risk factors) and subsequent exhaustion of the pool of progenitor cells. Although the definition and role of EPCs remains to be determined, we believe that aging of stem and/or progenitor cells contributes to the pathology of age-associated disease. As with mature vascular cells, EPCs seem to undergo senescence via both telomere-dependent and telomere-independent mechanisms.

Effect of senescence on function of endothelial progenitor cells

The telomere length of white blood cells has been shown to decline with advancing age in healthy individuals and more-rapid telomere shortening occurs in white blood cells from patients with CAD,[57] implying that age and coronary risk factors could influence telomere length in EPCs. Consistent with this notion, elderly individuals have a higher number of senescent EPCs than young subjects.[46] EPCs from individuals at high risk of cardiovascular events show a higher rate of senescence *in vitro* than do EPCs from individuals with a low cardiovascular risk, possibly because EPCs 'age' at a faster rate in patients at risk of cardiovascular events and these individuals therefore have a proportionally greater number of senescent EPCs than healthy persons.[49]

Progressive shortening of EPC telomeres in CAD patients with the metabolic syndrome is associated with increased oxidative stress rather than age.[58] Likewise, early onset of senescence and activation of the p53/p21 signaling pathway have been detected in EPCs from patients with diabetes.[59] Deletion of p53 inhibits senescence of EPCs from individuals with diabetes and restores the ability of these cells to form tube-like structures.[59] A number of *in vitro* experiments have suggested that senescence has a potential role in age-associated impairment of EPC function. Exposure of cultured EPCs to oxidized LDL induces dose-dependent functional impairment and accelerates EPC senescence, possibly by inactivation of telomerase.[60] An increase in angiotensin II levels also diminishes telomerase activity and accelerates the onset of EPC senescence through an increase in oxidative stress.[60] Both angiotensin-II-induced senescence and angiotensin-II-induced inhibition of telomerase activity could be blocked by antioxidants. Homocysteine is another powerful coronary risk factor that can inhibit telomerase activity, thereby accelerating the senescence of EPCs.[61]

Conversely, introduction of telomerase has been shown to enhance the regenerative and angiogenic ability of EPCs, increasing the therapeutic efficiency of these cells in a murine model of hind limb ischemia.[62] Statin therapy is reported to reduce senescence of isolated human EPCs by modulating cell-cycle regulators and telomere binding protein.[63,64] Estrogen also increases telomerase activity and thus inhibits EPC senescence.[6]

Role of cytokines in regulating function of endothelial progenitor cells

Humans exhibit an age-dependent decline in the production of angiogenic cytokines and growth factors that is related to age-associated impairment of EPC function. This decline in angiogenic stimuli is partly attributable to an impaired response to ischemic injury. For example, aging decreases the stability of hypoxia-inducible factor 1α (HIF-1α)—a transcription factor that mediates the adaptive response to hypoxia—resulting in decreased expression of stromal-cell-derived factor and vascular endothelial growth factor.[65,66] Introduction of a constitutively activated form of HIF-1α restores the response to hypoxia in aged animals and actually increases the number of circulating EPCs.[65] These angiogenic factors might have a key role in promoting the mobilization, migration and proliferation of EPCs, as well as in inhibiting senescence in these cells. Indeed, levels of insulin-like growth factor 1 are considerably reduced in elderly men compared with younger individuals, and this decline is associated with an increase in EPC dysfunction and in the proportion of senescent EPCs.[46] Treatment of elderly people with growth hormone increases the levels of insulin-like growth factor 1 and reduces EPC senescence, presumably via telomerase activation. Inhibition of EPC senescence by administration of growth factors could, therefore, be a novel therapeutic strategy for age-related vascular disorders.

PREMATURE AGING SYNDROMES

The term 'progeroid syndrome' includes a range of inherited disorders characterized by features of rapid aging. Among the human progeroid syndromes, Werner syndrome (WRN) and Hutchinson–Gilford progeria syndrome (HGPS) are two of the best characterized disorders and those that most closely mimic the features of human aging, including the cardiovascular changes.[67,68] Recently, major progress has been made in understanding the genetic, biochemical and cellular basis of these syndromes. Accumulating evidence indicates that the senescence of adult somatic cells and stem cells has a crucial role in the premature aging phenotype.

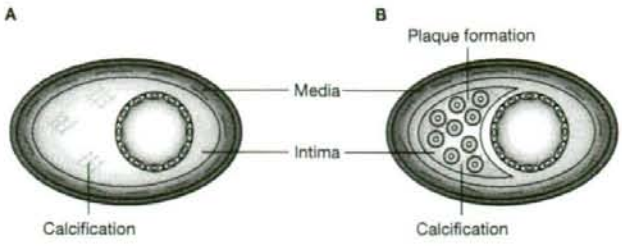
Hutchinson–Gilford progeria syndrome

HGPS is referred to as 'childhood progeria' to differentiate the disorder from WRN, which is referred to as 'adult progeria'. Initially, patients do

not have any cardiovascular problems, but gradually develop shortness of breath with exertion between 6 years and 8 years of age.[69] Mortality in patients with HGPS is frequently a result of myocardial infarction or cerebrovascular events, which occur at an average age of 13 years.[69] At autopsy, the major finding in the cardiovascular system is extensive loss of VSMCs from the medial layer of the aorta and other great vessels, as well as smaller arteries (Figure 3A).[69,70] Most strikingly, unlike normal human aging, atherosclerotic plaques are very rare.

The genetic basis of HGPS was discovered in 2003, when it was found that most children with the disease have a single-nucleotide substitution in the lamin A gene (*LMNA*)—encoding A-type nuclear lamins—that leads to aberrant splicing.[71,72] Lamin A is synthesized as a precursor protein (prelamin A), which is cleaved twice by specific enzymes, including CAAX prenyl protease 1 homolog (also known as zinc metalloproteinase Ste24 homolog [*ZMPSTE24*]), during which the farnesyl modification is removed. The most frequent HGPS-associated mutation, Gly608Gly, is a silent base-substitution that results in internal deletion of 50 amino acid residues from the C terminus of lamin A. The resulting protein cannot be processed by CAAX prenyl protease 1 homolog. The mutant lamin A—called progerin—remains farnesylated and anchored to the nuclear envelope.[72] Consequently, patients with HGPS display various cellular changes such as irregular nuclear morphology and disorganization of heterochromatin, both associated with abnormal regulation of gene expression.

Several murine models of HGPS have been developed and provide insight into the mechanism of this disease. Mounkes *et al.* created a mouse model expressing mutant *Lmna*, which causes Emery–Dreifuss muscular dystrophy in humans, and found that these mutant mice displayed a severe HGPS-like phenotype.[73] Another mouse model of HGPS, generated by transgenic expression of human *LMNA* that contained the Gly608Gly mutation, showed changes that were largely restricted to the vascular system.[74] These mice exhibited progressive loss of VSMCs in the media of large arteries, which is very similar to the changes seen in patients with HGPS. Such structural changes in these mice were associated with vascular dysfunction exemplified by a lack of vasodilator response. By contrast, *Zmpste24*-knockout



- Loss of VSMC in the media
- Acellular intima
- Replacement of VSMC by fibrous tissue
- Rare plaque formation

- Atherosclerotic plaque formation
- Frequent calcification

Figure 3 Histological features of progeroid vascular tissues. (**A**) Histological features of Hutchinson–Gilford progeria syndrome include extensive loss of VSMCs in the medial layer. Intimal thickening is present, but the intima is also acellular. Medial and intimal VSMCs are replaced by fibrous tissue and plaques rarely form. (**B**) Atherosclerotic plaque formation is common in individuals with Werner syndrome, and the plaques frequently show signs of calcification. Abbreviation: VSMCs, vascular smooth muscle cells.

mice have a broader progeria-like phenotype with severe growth retardation, skin defects and increased mortality.[75] Deletion of *Lmna* in mice results in a muscular-dystrophy-like phenotype that differs from mouse models of HGPS; therefore, permanent farnesylation of progerin seems to be implicated in the pathogenesis of HGPS.[68] This hypothesis is further supported by the finding that *Zmpste24*-knockout mice with reduced levels of farnesylated progerin have a normal phenotype.[76] Moreover, inhibiting farnesylation of prelamin A improves considerably the growth and survival of murine models of HGPS, and the shape of fibroblast nuclei is restored to normal.[68,77] Earlier this year, Varela *et al.* reported that prelamin A undergoes alternative prenylation by geranylgeranyltransferase upon inhibition of farnesyltransferase, which compromises the effects of farnesyltransferase inhibitors.[78] Consequently, inhibition of both farnesylation and geranylgeranylation of prelamin A with a combination of statins and aminobisphosphonates improves the aging-like phenotypes of *Zmpste24*-knockout mice.

Although the precise mechanisms by which progerin produces a progeria-like phenotype remain unclear, it seems likely that accumulation of progerin induces progressive changes to the nuclear architecture and exerts epigenetic control over the expression of genes that induce

premature aging.[79] In fact, progerin accumulates at later passages of HGPS fibroblasts and its accumulation is associated with loss of heterochromatin as well as progressive deformation of the nucleus.[80] Interestingly, fibroblasts from HGPS mice and fibroblasts from *Zmpste24*-knockout mouse embryos undergo premature cellular senescence as a consequence of increased DNA damage and exhibit genomic instability as a result of defects in the checkpoint response and DNA repair.[81] Deletion of p53 in *Zmpste24*-knockout mice improves some of the progeroid changes and extends life span,[82] suggesting that these cellular abnormalities are linked to the etiology of HGPS. Earlier this year, Scaffidi and Misteli demonstrated that expression of progerin activates downstream effectors of the Notch signaling pathways.[83] This activation causes dysregulation of stem-cell differentiation and possibly reduces cell life span. As lamin A mutations stimulate early apoptosis, it has also been suggested that high apoptotic cell death could deplete stem-cell pools, leading to impaired tissue regeneration.[84] These alterations could be involved in the development of vascular abnormalities; however, further studies are required to elucidate whether senescence of cardiovascular cells, including stem cells, accounts for the progeroid phenotype in patients with HGPS.

Werner syndrome

Patients with WRN are characterized by short stature, early graying and hair loss, an increased frequency of cancer, bilateral cataracts, scleroderma-like skin changes, type 2 diabetes, and atherosclerosis.[67,68] In contrast with individuals affected by HGPS, a considerable amount of atherosclerotic plaque accumulates in the coronary arteries and the aorta of patients with WRN, and calcium deposits are also observed in the aortic valve and mitral annulus (Figure 3B).[85] Patients with this syndrome usually die during middle age, with myocardial infarction and stroke being the two main causes. The mutation that causes WRN affects a member of the RecQ family of helicases called the *WRN* gene.[86] The WRN protein has helicase, exonuclease and single-stranded DNA annealing activities and is involved in DNA recombination, replication, repair and transcription, as well as in maintaining telomere integrity.[87]

Three different animal models of WRN have been developed to date: one has complete knockout of the WRN protein;[88] another transgenic expression of a human mutant WRN protein that lacks helicase activity;[89] and the third has in-frame deletion of the helicase domain.[90] WRN-protein-knockout mice develop normally and do not exhibit premature aging.[88] By contrast, WRN-protein-knockout mice that have shorter telomeres representative of the length in humans have similar phenotypic changes to those seen in patients with WRN, including graying and loss of hair, osteoporosis, diabetes and cataracts.[91,92] Telomere attrition is, therefore, a key element in the pathology of WRN. Fibroblasts from patients with WRN are known to undergo premature senescence.[67] Consistent with results obtained in WRN-protein-deficient mice with short telomeres, introduction of telomerase can extend the life span and reduce the genomic instability of these fibroblasts.[93,94]

Although the *in vivo* changes in the transgenic mouse model of WRN have not been completely described, cells derived from these mice grow less readily and are more sensitive to agents that cause DNA damage than are normal mice cells.[89] The third model of WRN, in which the helicase domain is deleted, exhibits increased genomic instability, telomere attrition, and an increased incidence of tumors.[90] Cells from these mice show premature loss of proliferative activity. When this model is back-crossed to an inbred strain, the resulting mice have abnormal increases in visceral fat with high fasting triglyceride and cholesterol levels, and subsequently develop insulin resistance and high blood glucose levels.[95] Although atherosclerosis has not been observed, back-crossed adult mice develop severe cardiac fibrosis and show an increase in reactive oxygen species, as well as oxidative DNA damage in cardiac tissues. These findings imply that in the absence of functional WRN protein, cells accumulate toxic DNA intermediates or undergo telomere shortening. These changes could trigger genetic instability and DNA damage, which in turn could increase the DNA mutation rate or cellular senescence. Accumulation of dysfunctional cells may underlie the pathology of WRN.

TREATMENT OF VASCULAR AGING: FUTURE PERSPECTIVES

Recent studies on human progeroid syndromes have provided considerable insight into the treatment of age-associated vascular disease.

Cellular aging signals are potential targets for the treatment of atherosclerosis, as a single gene mutation that induces premature cellular senescence could cause vascular abnormalities. Specific individualized therapies could be developed for each patient with premature vascular disease by searching for reagents that improve the senescence-like phenotype at the cellular level, similar to the action of farnesylation inhibitors.

Several lines of evidence suggest that telomere-dependent senescence underlies age-associated vascular pathophysiology, thus one candidate for antisenescence therapy of atherosclerosis is telomerase. In addition to telomerase itself, all molecules involved in regulating telomerase activity could be used as therapeutic tools. For example, estrogen increases expression of the catalytic component of telomerase, thereby inducing telomerase activity. A number of reports have demonstrated that telomerase is activated by medications known to exert a beneficial effect on cardiovascular disease, such as statins,[64] thiazolidinediones[96] and aspirin.[97] Treatment with these medications or with humoral factors could prevent progressive telomere shortening in vascular cells and delay the onset of age-associated vascular dysfunction. Angiotensin II type 1 receptor antagonists could also be useful for the treatment of vascular aging by suppressing angiotensin-II-induced senescence.[33] Antagonists to p53 are available,[98] but systemic inhibition of p53 activity could induce tumorigenesis. Vascular-specific regulation of cellular aging signals would, therefore, be required. Recent studies indicate that tissue-specific inhibition of the signaling pathways for senescence increases longevity in a non-cell-autonomous manner.[99] Consequently, it would be interesting to investigate whether cellular aging signals in certain tissues regulate those in the vasculature.

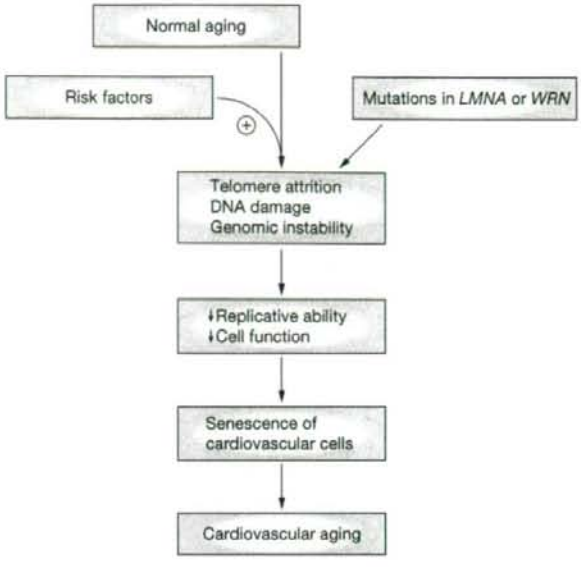


Figure 4 Potential common pathways for normal aging and age-associated cardiovascular disease. Telomere attrition, DNA damage and genomic instability are increased in the elderly and in patients with progeroid syndromes, thereby inducing senescence of cardiovascular cells including stem and/or progenitor cells. Risk factors for cardiovascular disease override the effect of chronological aging on cell turnover by accelerating stress-induced damage. Senescence of cardiovascular cells is associated with decreased replication and cellular dysfunction that contribute to the development of cardiovascular aging. Abbreviations: *LMNA*, lamin A gene; *WRN*, Werner syndrome gene.

CONCLUSIONS

Cell division is essential for the survival of multicellular organisms that contain renewable tissues, but places the organism at risk of developing cancer. Thus, complex organisms have evolved at least two cellular mechanisms to prevent oncogenic transformation—apoptosis and cellular senescence. In this regard, aging and age-associated diseases can be viewed as by-products of the tumor suppressor mechanism known as cellular senescence. Consistent with this idea, the number of senescent fibroblasts increases exponentially in the skin of aging primates.[16] Conversely, extension of life span by calorie restriction decreases biomarkers of cellular senescence *in vivo*.[160] In human progeroid syndromes, DNA damage signaling pathways are activated, thereby promoting premature senescence and apoptosis; accumulation of senescent cells and excessive cell death is thought to contribute to the pathogenesis of these syndromes. As discussed, the features of premature aging (including cardiovascular changes) in patients with progeroid syndromes are markedly different to those of normal aging. Indeed, it is not clear whether WRN and HGPS polymorphisms are associated with human aging or age-associated cardiovascular disease. Features common to progeroid syndromes and normal aging are, however, likely to exist (Figure 4), particularly at the cellular level. Identification of such traits could lead to new treatment strategies for cardiovascular disease as well as for aging.

KEY POINTS

- Cellular senescence probably contributes to the pathogenesis of cardiovascular disease
- Telomere integrity is impaired with advancing age, which leads to vascular dysfunction
- The number and function of cardiovascular stem cells and/or progenitor cells shows a progressive decline with age
- Telomere attrition, DNA damage, and genomic instability might be common features of both normal aging and progeroid syndromes

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Role of Heat Shock Transcriptional Factor 1 and Heat Shock Proteins in Cardiac Hypertrophy

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Cardiac hypertrophy is an independent risk factor for cardiovascular disease. Initially, cardiac hypertrophy is an adaptive response to increased wall stress, but sustained stress leads to heart failure. It remains unclear how the transition from adaptive cardiac hypertrophy to maladaptive cardiac hypertrophy occurs. It has been postulated that there are two forms of cardiac hypertrophy, which are physiologic and pathologic cardiac hypertrophy. Unlike pathologic cardiac hypertrophy caused by chronic pressure or volume overload, cardiac hypertrophy induced by exercise is associated with less fibrosis and better systolic function, suggesting that adaptive mechanisms may be involved in exercise-induced cardiac hypertrophy. Therefore, elucidation of the molecular differences between these two types of cardiac hypertrophy may provide insights into the mechanisms underlying the transition from adaptive cardiac hypertrophy to heart failure. By comparing the two types of cardiac hypertrophy, we have identified heat shock transcription factor 1 and its target heat shock proteins as key factors involved in the adaptive mechanism of cardiac hypertrophy. In this review, we summarize the protective role of heat shock transcription factor 1 and heat shock proteins in cardiovascular disease.
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• Introduction

Heart failure is the final outcome of various heart diseases, and cardiac hypertrophy is one of the main causes of heart failure. The Framingham Heart Study revealed that there is a relationship between the severity of cardiac hypertrophy and the incidence of cardiovascular events, and that cardiac hypertrophy is an independent risk factor for heart failure, arrhythmia, myocardial infarction, and sudden death (Levy et al. 1990, Behar et al. 1992, Haider et al. 1998, Verdecchia et al. 2001). Therefore, it is important to develop therapeutic strategies for this condition, but the precise mechanisms underlying the transition from cardiac hypertrophy to heart failure are still largely unknown.

Cardiac hypertrophy is induced by various pathologic or physiologic stimuli. For example, acute pressure overload initially induces adaptive cardiac hypertrophy that is associated with normal cardiac function, but systolic and diastolic dysfunction occur in the setting of chronic pressure overload, resulting in heart failure. Thus, chronic pressure overload is thought to cause pathologic or maladaptive cardiac hypertrophy. On the other hand, regular exercise can induce cardiac hypertrophy without causing systolic or diastolic dysfunction (Pluim et al. 2000). Because exercise-induced cardiac hypertrophy does not progress to heart failure, it is thought to be physiologic or adaptive cardiac hypertrophy. Although it has been reported that these two types of cardiac hypertrophy are morphologically (Richey and Brown 1998, Iemitsu et al. 2001, McMullen and Jennings 2007), functionally, and molecularly distinct from each other, the precise mechanism underlying these differences remains unclear. What are the exact differences between pathologic and physiologic cardiac hypertrophy? Why is cardiac function preserved in physiologic cardiac hypertrophy? Why does sustained pressure overload cause heart failure? Answering these questions will provide insights into novel therapeutic options for both cardiac hypertrophy and heart failure.

• Pathologic and Physiologic Cardiac Hypertrophy

The differences between these two conditions include the stimuli inducing cardiac hypertrophy, their duration of action, and the signaling pathways involved. Pathologic cardiac hypertrophy is induced by persistent stress, such as pressure overload and volume overload caused by hypertension or valvular heart disease. On the other hand, physiologic cardiac hypertrophy is induced by intermittent stress such as exercise. Thus, the manifestations of cardiac hypertrophy caused by various stimuli may depend on their duration and intensity. In a recent study, Perrino et al. (2006) applied intermittent pressure overload to the heart and investigated the role of the duration of stress in the development of cardiac failure. Despite only developing mild cardiac hypertrophy, the hearts exposed to intermittent pressure overload displayed various pathologic changes, including diastolic dysfunction and histologic abnormalities.

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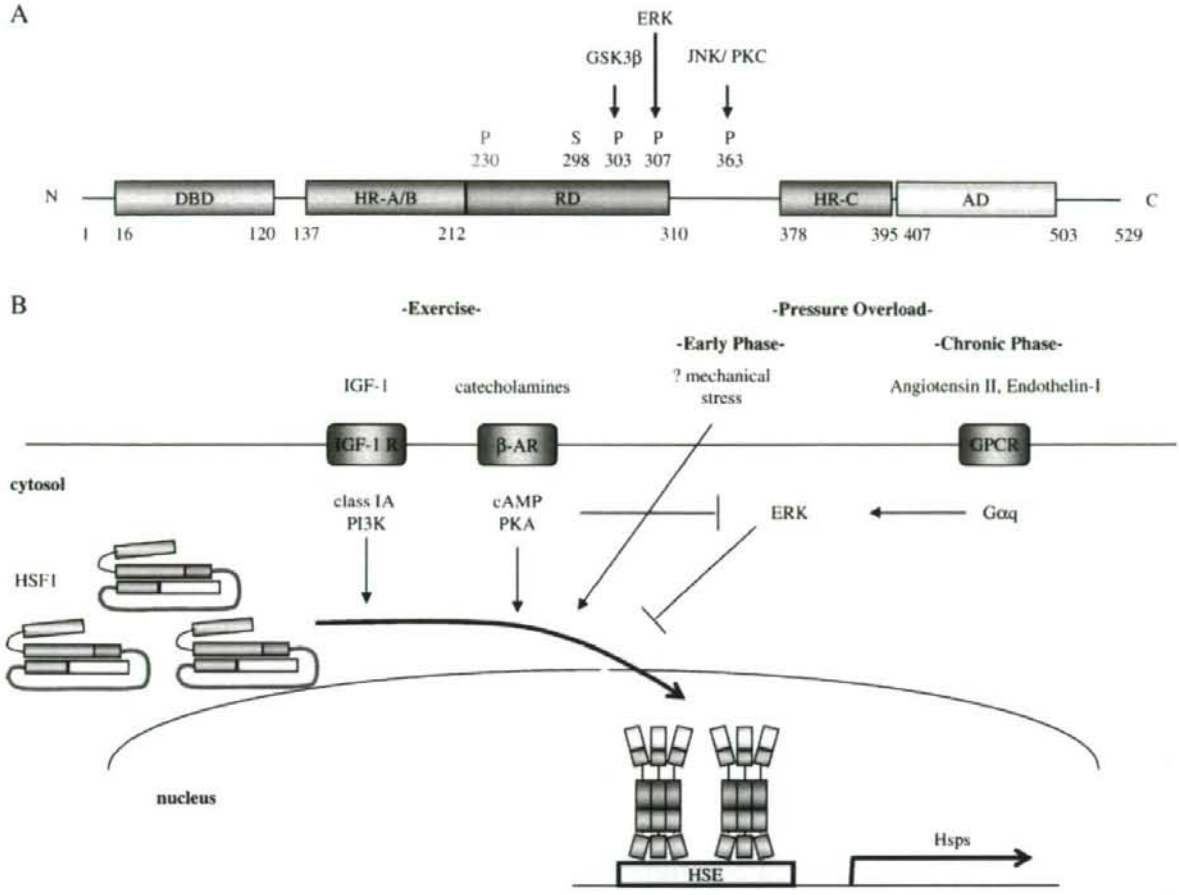

Figure 1. Potential regulators of HSF1 in cardiac hypertrophy. (**A**) Structure of HSF1. DBD indicates DNA-binding domain; HR, hydrophobic repeat; RD, regulatory domain; AD, transcriptional activation domain; P, phosphorylated site (the activating site is indicated in red); S, sumoylated site. (**B**) Potential regulatory mechanism of HSF1. Under nonstressful conditions, HSF1 exists as a monomer whose transcriptional activity is repressed by phosphorylation of the repressing sites (Ser303, Ser307, and Ser363). Upon stress, phosphorylation of the activating site (Ser230) is enhanced, thereby promoting the transcriptional activity of the trimerized and DNA-bound HSF1. The ratio of phosphorylation between the activating and repressing sites may be influenced by various stimuli, such as IGF-1, catecholamine, and angiotensin II, and determine the magnitude of the transcriptional activity. IGF-1R indicates IGF-1 receptor; β-AR, β adrenergic receptor; GPCR, G-protein-coupled receptor.

Thus, the nature of the stress acting on the heart, rather than its duration, may be a key determinant of the maladaptive phenotype.

A number of studies have shown that various signaling pathways contribute to the development of pathologic and physiologic cardiac hypertrophy by using mice that overexpress or lack specific genes (Richey and Brown 1998, Selvetella et al. 2004, Heineke and Molkentin 2006, Shiojima and Walsh 2006, McMullen and Jennings 2007). Endocrine factors such as angiotensin II and endothelin 1 induce pathologic cardiac hypertrophy (Yamazaki et al. 1995, Yamazaki et al. 1996), whereas inhibition of angiotensin II by angiotensin-converting enzyme inhibitors or angiotensin II receptor type 1 blockers can lead to regression of cardiac hypertrophy (Okin et al. 2003). Overexpression of Gαq in the heart, which is activated by these factors, also induces cardiac hypertrophy associated with cardiac dysfunction (D'Angelo et al. 1997), whereas overexpression of an inhibitory peptide that interferes with Gαq coupling prevents the onset of maladaptive cardiac hypertrophy (Akhter et al. 1998). These findings suggest that the Gαq-mediated pathway is important for the development of pathologic cardiac hypertrophy.

The calcium/calmodulin-dependent phosphatase calcineurin has also been suggested to have a role in pathologic cardiac hypertrophy. Transgenic mice that overexpress active forms of calcineurin or its downstream transcription factor (NFAT3) develop cardiac hypertrophy and heart failure (Molkentin et al. 1998). Calcineurin inhibitors, such as cyclosporin A and FK506, suppress angiotensin II-induced cardiomyocyte hypertrophy in vitro and inhibit pressure overload-induced cardiac hypertrophy in vivo (Molkentin et al. 1998, Shimoyama et al. 2000). Overexpression of a dominant-negative mutant of calcineurin in

the heart also suppresses the induction of pathologic cardiac hypertrophy by pressure overload (Zou et al. 2001).

On the other hand, it has been reported that the insulin-like growth factor-1 (IGF-1)/class I_A phosphoinositide 3-kinase (PI3K) pathway is activated in physiologic cardiac hypertrophy. Cardiac production of IGF-1 is significantly higher in athletes than in control subjects (Neri Serneri et al. 2001, Melling et al. 2006), and serum levels of IGF-1 increase in response to training (Koziris et al. 1999). Transgenic mice overexpressing the IGF-1 receptor or a constitutively active form of class I_A PI3K in the heart develop cardiac hypertrophy without cardiac dysfunction or an increase of fibrosis (Shioi et al. 2000, McMullen et al. 2004). In contrast, transgenic mice with reduced cardiac class I_A PI3K activity have smaller hearts and show a blunted hypertrophic response to exercise training, but not to pressure overload (McMullen et al. 2003, Luo et al. 2005). These results suggest that the IGF-1/class I_A PI3K pathway is involved in the regulation of cardiac growth during postnatal development, and that this pathway plays a crucial role in inducing physiologic cardiac hypertrophy.

Although there have been a number of previous reports about the stimuli and signaling pathways involved in the regulation of physiologic or pathologic cardiac hypertrophy, the target genes and molecules of these pathways remain unclear. To answer these questions, various research groups have compared the pattern of cardiac gene expression between physiologic and pathologic cardiac hypertrophy (Richey and Brown 1998, Iemitsu et al. 2001, McMullen and Jennings 2007). These studies have shown that an array of genes display differential expression, suggesting that such differences might be involved in producing the two distinct phenotypes of cardiac hypertrophy. However, it remains to be determined whether these gene products actually promote different types of cardiac hypertrophy. Recently, we examined gene expression patterns in the heart and found differences in the expression of about 100 genes between physiologic and pathologic cardiac hypertrophy. Among them, we examined the role of heat shock proteins (HSPs) and heat shock transcription factor 1 (HSF1) in cardiac hypertrophy because the expression of *Hsp70* and *Hsp27* was only elevated in physiologic cardiac hypertrophy.

- **Role of Heat Shock Transcriptional Factor 1/HSPs in Cardiovascular Disease**

Heat shock proteins are ubiquitously expressed, and their expression is enhanced by various acute and chronic stimuli, such as heat shock, heavy metals, low molecular weight toxins, infection, and oxidative stress (Li and Laszlo 1985, Benjamin and McMillan 1998, Morimoto 1998, Pockley 2002, Westerheide and Morimoto 2005). Heat shock proteins act to ensure the proper protein folding, as well as to prevent protein misfolding and assist in protein refolding to the correct state. Expression of HSPs is mainly regulated by HSF1 at the transcriptional level. In the unstressed state, HSF1 exists as a latent monomer, with repressed DNA binding and transcriptional activity. Upon activation, HSF1 undergoes multiple processes that include a monomer-to-trimer transition, nuclear accumulation, binding to the heat shock element located in the promoter region of each HSP gene, and transcriptional activation (Figure 1). Heat shock transcription factor 1–heat shock element DNA binding is not sufficient to elicit maximal transcription of the HSP genes, and it is necessary for HSF1 to be modified by phosphorylation and sumoylation to increase its transcriptional activity (Holmberg et al. 2002, Westerheide and Morimoto 2005). It has been suggested that HSF1 is repressed by GSK-3β (Ser303), ERK (Ser307), and JNK (Ser363) under normal conditions, whereas it is activated by hyperphosphorylation (Ser-230) upon exposure to various stresses (Figure 1A) (Chu et al. 1996, Chu et al. 1998, Morimoto 1998, Holmberg et al. 2002). However, the mechanisms underlying the activation of HSF1, particularly its regulation by phosphorylation, remain unclear.

A number of studies have shown that HSF1 and HSPs confer protection against cardiovascular disease. Induction of HSF1 and HSP expression by various stimuli, such as heat shock, reduces the size of infarcts after ischemia/reperfusion (Donnelly et al. 1992, Marber et al. 1993, Bennani et al. 1998). Transgenic mice overexpressing a constitutively active form of HSF1 or inducible Hsp70 in the heart show more resistance to ischemia/reperfusion injury compared with wild-type mice (Marber et al. 1995, Plumier et al. 1995, Zou et al. 2003). In contrast, the cardiac function of inducible Hsp70 knockout mice is markedly impaired by ischemia/reperfusion injury (Kim et al. 2006). In addition to a protective effect against ischemia/reperfusion injury, it has been reported that HSPs have a beneficial role in myocardial infarction, doxorubicin-induced cardiomyopathy, and atrial fibrillation (Baljinnyam et al. 2006, Brundel et al. 2006, Venkatakrishnan et al. 2006, Liu et al. 2007, Wakisaka et al. 2007).

Our recent study identified HSF1 as a critical transcription factor that regulates cardiac hypertrophy (Sakamoto et al. 2006). Heat shock transcription factor 1 was only activated in exercise-induced cardiac hypertrophy, but not in chronic pressure overload-induced cardiac hypertrophy. When heterozygous $HSF1^{+/-}$ mice (Inouye et al. 2004) were forced to exercise (which is thought to induce physiologic cardiac hypertrophy), significant systolic dysfunction occurred. In contrast, when transgenic mice that expressed a constitutively active form of HSF1 (Nakai et al. 2000) were exposed to chronic pressure overload (which is thought to induce pathologic cardiac hypertrophy), their systolic function was preserved. These results indicate that HSF1 is a key molecule for preservation of systolic function during the development of cardiac hypertrophy under both pathologic and physiologic conditions. Accumulation and aggregation of unfolded proteins are associated with an increase of protein synthesis in hypertrophied hearts and induce cardiomyocyte death that eventually leads to systolic dysfunction (Okada et al. 2005). Thus, the protective effects of HSF1 may be attributable to the functions of HSPs in protein folding and degradation. In addition to such well-known functions, accumulating evidence indicates that different HSPs directly act on the cell death machinery and inhibit the signaling pathway for cell death at various points (Sreedhar and Csermely 2004). For example, Hsp27 binds to cytochrome c and prevents it from binding to Apaf-1 (Bruey et al. 2000), whereas Hsp70 prevents Apaf-1 from recruiting procaspase-9 (Beere et al. 2000), thereby inhibiting apoptotic cell death. It is

conceivable that sustained activation of HSF1 prevents the onset of cardiac dysfunction in hypertrophic hearts through the mechanisms involving a direct action of HSPs on the cell death machinery as well as their functions in protein degradation.

• Potential Regulators of HSF1 in Cardiac Hypertrophy

Heat shock transcription factor 1 and HSPs are upregulated by exercise (Taylor et al. 1999, Hamilton et al. 2001, Sakamoto et al. 2006), but the mechanisms involved are not fully understood. As mentioned above, the IGF-1/class I_A PI3K pathway is thought to play an important role in inducing physiologic cardiac hypertrophy (McMullen et al. 2004). Interestingly, expression of HSPs is increased in the hearts of transgenic mice, with enhancement of cardiac IGF-1 or class I_A PI3K, suggesting a potential relationship between this signaling pathway and HSF1 activity. Consistent with this notion, the IGF-1/class I_A PI3K pathway is known to inhibit GSK-3β (Shiojima and Walsh 2006), which is a negative regulator of HSF1. It could be assumed that IGF-1-induced inhibition of GSK-3β contributes to the activation of HSF1 in exercise-induced cardiac hypertrophy (Figure 1B).

Another possibility is that catecholamines may upregulate HSF1 and HSPs after exercise, because circulating levels of catecholamines are increased by exercise. Isoproterenol (a β-adrenergic agonist) increases cardiac expression of HSP70 (White and White 1986), whereas inhibition of protein kinase A (PKA), a downstream kinase of the β-adrenergic receptor, suppresses exercise-induced upregulation of *Hsp70* (Melling et al. 2004). Moreover, exercise-induced activation of PKA attenuates the phosphorylation of ERK, which is a negative regulator of HSF1 (Melling et al. 2006). Taken together, these findings suggest that exercise may upregulate HSF1 by activating the β-adrenergic signaling pathway that induces PKA-mediated inactivation of ERK (Figure 1B). Although activation of protein kinase C in the heart during exercise is thought to have a protective role, it remains unclear whether this pathway is involved in the upregulation of HSF1 and HSPs after exercise (Yamashita et al. 2001, Melling et al. 2004). Moreover, posttranslational modifications rather than phosphorylation may regulate the transcriptional activity of HSF1 during exercise.

Our findings showed that HSF1 was only activated in the early phase of pressure overload (the adaptive phase), but not in the chronic phase (the maladaptive phase) (Sakamoto et al. 2006). Other groups have also demonstrated that acute pressure overload activates HSF1 and increases the expression of HSPs (Delcayre et al. 1988, Izumo et al. 1988, Nishizawa et al. 2002). Why is HSF1 downregulated during the chronic phase of pressure overload? Production of autocrine/paracrine factors such as angiotensin II and endothelin 1 is increased by pathologic stimuli and plays a critical role in inducing pathologic cardiac hypertrophy. These factors bind to G-protein-coupled receptors, leading to dissociation of the Gαq subunit and activation of downstream signaling molecules, which include negative regulators of HSF1 such as ERK and JNK. Accordingly, this signaling pathway may induce pathologic cardiac hypertrophy partly via the inactivation of HSF1 (Figure 1B), although there is a conflicting report that angiotensin II does not influence the activity of HSF1 (Nishizawa et al. 2002). Further studies are necessary to elucidate precisely how HSF1 activity is regulated as cardiac hypertrophy develops.

• Conclusion and Future Prospects

Because there have been many reports that induction of HSF1 and HSPs has a beneficial effect in animal models of cardiovascular disease, activation of HSF1 and HSPs could be a novel therapeutic strategy for various cardiovascular diseases. Geranylgeranylacetone, an anti-ulcer agent, has been reported to upregulate HSF1 and HSPs, and shows a protective effect against ischemia/reperfusion injury and atrial fibrillation (Yamanaka et al. 2003, Brundel et al. 2006, Wakisaka et al. 2007). Exercise also upregulates HSF1 and HSPs, and it ameliorates cardiac dysfunction in hypertensive animals (Scheuer et al. 1982, Schaible et al. 1986, Moreno Junior et al. 1995, Emter et al. 2005). Moreover, recent studies have further demonstrated the protective effect of exercise on cardiac function in animal models of myocardial infarction and ischemia/reperfusion injury (Hoshida et al. 2002). However, conflicting data also suggest that any increase of HSPs in the heart after exercise is not necessary for protection against ischemia/reperfusion injury and that moderate exercise does not improve cardiac dysfunction in hypertensive rats (Taylor et al. 1999, Hamilton et al. 2001). Moreover, excessive exercise accelerates the rate of progression from cardiac hypertrophy to heart failure in untreated hypertensive rats (Sarma and Schulze 2007). To develop a novel therapeutic strategy targeting the HSF1/HSP system for patients with cardiovascular disease, one is required to perform further studies of elucidating the protective mechanisms involved.

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Understanding Proteasome Assembly and Regulation: Importance to Cardiovascular Medicine

Glen W. Young, Yueju Wang, and Peipei Ping*

The cardiac proteasome is increasingly recognized as a complex, heterogeneous, and dynamic organelle contributing to the modulation of cardiac function in health and diseases. The emerging picture of the proteasome system reveals a highly regulated and organized molecular machine integrated into multiple biologic processes of the cell. Full appreciation of its cardiovascular relevance requires an understanding of its proteolytic function as well as its underlying regulatory mechanisms, of which assembly, stoichiometry, posttranslational modification, and the role of the associating partners are increasingly poignant. (Trends Cardiovasc Med 2008;18:93–98) *Published by Elsevier Inc.*

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• Introduction

The mammalian protein degradation machinery is dominated by the proteasome, as it endoproteolytically cleaves more than 70% of intracellular proteins (Rock et al. 1994). The core of this multimeric protease is a duplex of two sets of 14 subunits, housing duplicate sites of trypsin-like, caspase-like, and chymotrypsin-like peptidase activities. Termed the *20S proteasome*, its gated pores maintain the complex in a latently active state, enabling only limited

Vascular Endothelial Growth Factor Receptor-1 Regulates Postnatal Angiogenesis Through Inhibition of the Excessive Activation of Akt

Jun-ichiro Nishi,* Tohru Minamino,* Hideyuki Miyauchi, Aika Nojima, Kaoru Tateno, Sho Okada, Masayuki Orimo, Junji Moriya, Guo-Hua Fong, Kenji Sunagawa, Masabumi Shibuya, Issei Komuro

Abstract—Vascular endothelial growth factor (VEGF) binds both VEGF receptor-1 (VEGFR-1) and VEGF receptor-2 (VEGFR-2). Activation of VEGFR-2 is thought to play a major role in the regulation of endothelial function by VEGF. Recently, specific ligands for VEGFR-1 have been reported to have beneficial effects when used to treat ischemic diseases. However, the role of VEGFR-1 in angiogenesis is not fully understood. In this study, we showed that VEGFR-1 performs "fine tuning" of VEGF signaling to induce neovascularization. We examined the effects of retroviral vectors expressing a small interference RNA that targeted either the VEGFR-1 gene or the VEGFR-2 gene. Deletion of either VEGFR-1 or VEGFR-2 reduced the ability of endothelial cells to form capillaries. Deletion of VEGFR-1 markedly reduced endothelial cell proliferation and induced premature senescence of endothelial cells. In contrast, deletion of VEGFR-2 significantly impaired endothelial cell survival. When VEGFR-1 expression was blocked, VEGF constitutively activated Akt signals and thus induced endothelial cell senescence via a p53-dependent pathway. VEGFR-$1^{+/-}$ mice exhibited an increase of endothelial Akt activity and showed an impaired neovascularization in response to ischemia, and this impairment was ameliorated in VEGFR-$1^{+/-}$ Akt$1^{+/-}$ mice. These results suggest that VEGFR-1 plays a critical role in the maintenance of endothelial integrity by modulating the VEGF/Akt signaling pathway. (*Circ Res.* **2008;103:261-268.**)

Key Words: VEGF ■ Akt ■ senescence ■ p53

Angiogenesis involves the differentiation, proliferation, and migration of endothelial cells, leading to tubulogenesis and the formation of vessels.[1] One of the most important receptors for angiogenesis is the vascular endothelial growth factor (VEGF) receptor, which is a member of the receptor tyrosine kinase family.[2,3] VEGF receptor (VEGFR)-1 and VEGFR-2 are closely related receptor tyrosine kinases and have both common and specific ligands. VEGFR-1 has weaker kinase activity, whereas VEGFR-2 is a highly active kinase that stimulates a variety of signaling pathways and induces a broad range of biological responses. Despite its weak kinase activity, VEGFR-1 is essential for normal development and angiogenesis.[4] VEGFR-1 null mutant mice die in utero because of the overgrowth of endothelial cells and vascular disorganization.[5,6] In contrast, mice expressing the VEGFR-1 that lacks the tyrosine kinase domain develop a normal cardiovascular system,[7] suggesting that VEGFR-1 kinase activity might not be required for vascular development during embryogenesis and that VEGFR-1 may act as a decoy receptor. Consistent with this concept, selective activation of chimeric VEGFR-1 (in the absence of chimeric VEGFR-2)[8] or a VEGF mutant that binds to VEGFR-1 does not influence cell proliferation, migration, or survival in vitro.[9–11]

However, recent studies have demonstrated that the role of VEGFR-1 in postnatal angiogenesis is more complicated than was initially recognized. For example, treatment with placenta growth factor (PlGF), a specific ligand for VEGFR-1, was reported to promote angiogenesis in vitro[11,12] and in vivo.[13] Overexpression of PlGF also induced angiogenesis in tumors[14] and the skin.[15] It has been suggested that stimulation by PlGF induces the heterodimerization of VEGFR-1 with VEGFR-2, leading to transactivation of VEGFR-2 and the promotion of angiogenesis.[8,16,17] Another possible explanation for the positive effect of PlGF on angiogenesis is that it prevents VEGF from binding to VEGFR-1, thereby

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increasing the binding and activation of VEGFR-2. In other studies, PlGF was shown to protect against hyperoxic vascular damage in the retina without provoking retinal neovascularization.[18] These results suggest that VEGFR-1 can either positively or negatively regulate angiogenesis depending on the circumstances, but further studies are required to better understand the role of this receptor in postnatal angiogenesis.

In the present study, we examined the effects of VEGFR-1 deletion on angiogenesis by using the retroviral vector expressing a small interference RNA that targeted the VEGFR-1 gene. Deletion of VEGFR-1 markedly reduced endothelial cell proliferation and thus impaired angiogenesis. Likewise, VEGFR-1$^{+/-}$ mice exhibited an impaired neovascularization in response to ischemia. This impairment was restored by inhibiting the excessive activation of Akt by VEGF. These results suggest that VEGFR-1 plays a critical role in the maintenance of endothelial integrity by modulating the VEGF/Akt signaling pathway.

Materials and Methods

Short Hairpin Interference RNA Vectors

The mammalian retrovirus expression vector pSIREN-RetroQ (Clontech) was used to achieve the expression of short hairpin interference RNA (shRNA) in human endothelial cells.

Statistical Analysis

Data are shown as mean±SEM. Differences between groups were examined by Student *t* test or ANOVA followed by the Bonferroni procedure for comparison of means. Values of $P<0.05$ were considered statistically significant.

Results

Effect of VEGF Receptor Gene Silencing on Endothelial Cell Function

To elucidate the role of VEGFR-1 in angiogenesis, we constructed mammalian retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1) or the VEGFR-2 gene (shVEGFR-2). Northern blot and Western blot analyses revealed that introduction of each construct into human umbilical vein endothelial cells caused effective and stable downregulation of the expression of the target molecule (Figure 1A and 1B, and supplemental Figure IA [available online at http://circres.ahajournals.org]). It is noted that either shVEGFR-1 or shVEGFR-2 did not affect VEGFR-2 or VEGFR-1 expression, respectively (Figure 1B, and supplemental Figure IA). We used two kinds of constructs for the following experiments and both of them achieved similar results. The nonsilencing control vector (shNega) was used as a control. After infected endothelial cells were purified by incubation with antibiotics, we performed the tube formation assay. Deletion of VEGFR-1 or VEGFR-2 significantly impaired tube formation compared with control cells (Figure 1C). We next examined the proliferative activity of infected cells. We seeded 2×10^5 infected cells into 100-mm dishes with VEGF-A on day 0 and counted cell number on day 3. Compared with shNega-infected control endothelial cells, both shVEGFR-1– and shVEGFR-2–infected cells showed significantly lower proliferation (Figure 1D). Deletion of VEGFR-1 caused more marked impairment of cell proliferation than deletion of VEGFR-2 (Figure 1D). This inhibitory effect of VEGFR-1 deletion was more evident when infected endothelial cells were subjected to long-term culture. Although VEGFR-2 deletion slightly reduced the lifespan of cells compared with that of control cells, VEGFR-1 deletion significantly shortened the lifespan of endothelial cells (Figure 1E). As a result, shVEGFR-1–infected cells underwent irreversible growth arrest earlier than shVEGFR-2-infected cells (Figure 1E). After growth arrest, the cells exhibited characteristics of senescence, becoming flatter and larger and showing an increase of senescence-associated β-galactosidase activity (Figure 1F). These findings suggest that VEGFR-1 deletion induces premature endothelial cell senescence. We next examined the effect of VEGFR-1 deletion on endothelial survival. We cultured infected cells in regular growth medium for 24 hours and subsequently cultured the cells under serum-free conditions with VEGF-A. After 24 hours, the number of viable cells was counted. As compared with the viability of control cells, deletion of VEGFR-2, but not VEGFR-1, markedly decreased cell viability (Figure 1G). Consistent with these findings, activation of caspase 3 was detected in cells with VEGFR-2 deletion, but not VEGFR-1 deletion (Figure 1H). These results suggest that VEGFR-1 is involved in the regulation of angiogenesis by regulating endothelial cell proliferation and senescence, whereas VEGFR-2 may be crucial for endothelial survival as well as cell proliferation.

VEGFR-1 Deletion Induces Endothelial Dysfunction by Activating Akt

To investigate the molecular mechanisms of premature senescence induced by VEGFR-1 deletion, we examined the transcriptional activity of p53 and its target gene p21. We transfected VEGFR-1–deleted endothelial cells with the luciferase reporter gene containing 13 copies of the p53-binding consensus sequence (pPG13-Luc). Deletion of VEGFR-1 significantly induced p53 transcriptional activity compared with that in shNega-infected cells, whereas VEGFR-2 deletion had no effect (Figure 2A). Likewise, p21 expression was significantly higher in VEGFR-1–deleted endothelial cells than in control cells or VEGFR-2–deleted cells (Figure 2B). However, expression of bax, another target molecule regulated by p53, was not altered in VEGFR-1–deleted endothelial cells compared with control cells (supplemental Figure IB). Ablation of p53 by the introduction of HPV16 E6 oncoprotein abolished the inhibitory effect of VEGFR-1 deletion on cell proliferation (Figure 2C). These results suggest that VEGFR-1 deletion induces endothelial cell senescence via a p53-dependent pathway.

We have previously demonstrated that Akt negatively regulates the endothelial cell lifespan by activating the p53/p21 pathway.[19] It has also been shown that Akt plays a central role in the regulation of angiogenesis by VEGF.[20] Thus, we examined the level of phosphorylated Akt in VEGFR-1-deleted endothelial cells. Western blot analysis

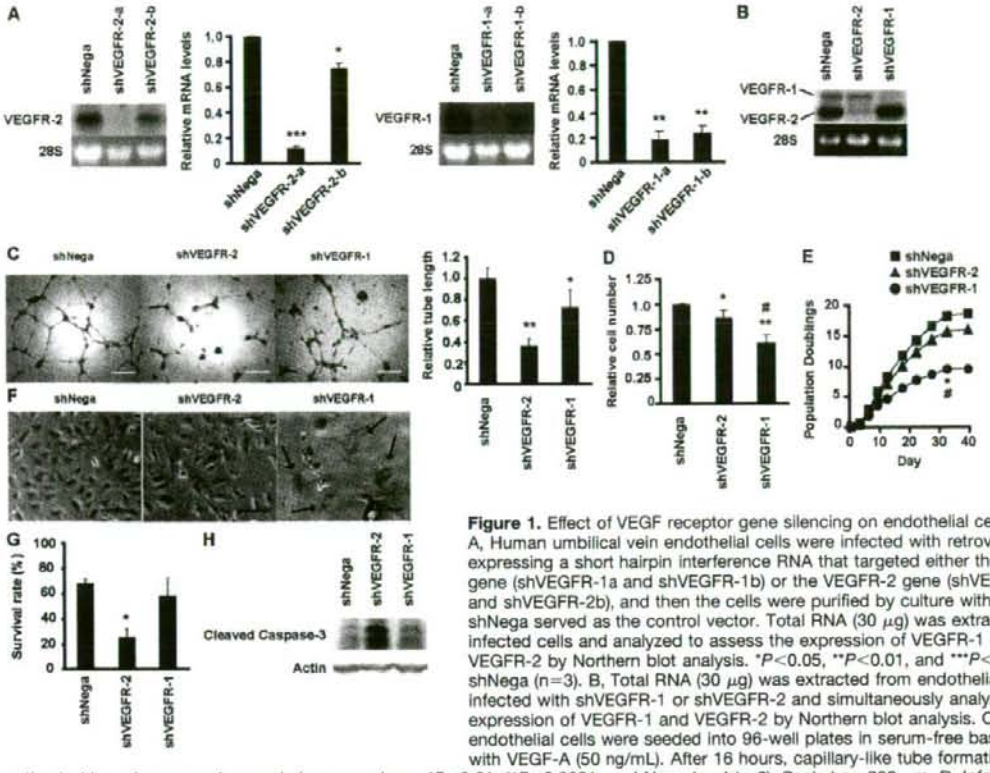

Figure 1. Effect of VEGF receptor gene silencing on endothelial cell function. A, Human umbilical vein endothelial cells were infected with retroviral vectors expressing a short hairpin interference RNA that targeted either the VEGFR-1 gene (shVEGFR-1a and shVEGFR-1b) or the VEGFR-2 gene (shVEGFR-2a and shVEGFR-2b), and then the cells were purified by culture with antibiotics. shNega served as the control vector. Total RNA (30 μg) was extracted from infected cells and analyzed to assess the expression of VEGFR-1 or VEGFR-2 by Northern blot analysis. $^*P<0.05$, $^{**}P<0.01$, and $^{***}P<0.001$ vs shNega (n=3). B, Total RNA (30 μg) was extracted from endothelial cells infected with shVEGFR-1 or shVEGFR-2 and simultaneously analyzed the expression of VEGFR-1 and VEGFR-2 by Northern blot analysis. C, Infected endothelial cells were seeded into 96-well plates in serum-free basic medium with VEGF-A (50 ng/mL). After 16 hours, capillary-like tube formation was estimated by using an angiogenesis image analyzer. $^*P<0.01$, $^{**}P<0.0001$ vs shNega (n=4 to 6). Scale bar: 300 μm. D, Infected endothelial cells were seeded at a density of 2×10^5 cells per 100-mm dish and cultured with VEGF-A (day 0). Then cell number was counted on day 3. $^*P<0.001$, $^{**}P<0.0001$ vs shNega, $\#P<0.001$ vs shVEGFR-2 (n=13 to 14). E, Infected cell populations were passaged until cells underwent senescence, and the total number of population doublings was determined. $^*P<0.01$ vs shNega, $\#P<0.05$ vs shVEGFR-2 (n=4 to 6). F, Morphology and senescence-associated β-galactosidase staining (arrow) of endothelial cells infected with shNega, shVEGFR-1, or shVEGFR-2. Scale bar: 100 μm. G, Infected endothelial cells were seeded at the density of 1×10^5 cells per 60-mm dish and cultured for 24 hours in growth medium. After washing twice with PBS, the cells were cultured in serum-free DMEM with VEGF-A (10 ng/mL). After 24 hours of serum starvation, the number of viable cells and the total number of cells were counted by a hemocytometer. $^*P<0.0001$ vs shNega (n=4 to 6). H, The lysates were extracted from cells, which are prepared as described in legend for G, and analyzed for cleaved caspase-3 expression by Western blotting.

showed that VEGFR-1 deletion led to a marked increase of the phosphorylated Akt level compared with that in control cells or cells with VEGFR-2 deletion, even under serum-free conditions (Figure 3A). VEGFR-1 deletion increased pAkt levels even in the absence of VEGF, presumably attributable to autocrine VEGF signaling (Figure 3B). Treatment with VEGF markedly increased pAkt levels within 5 to 15 minutes in VEGFR-1–deleted cells but not in VEGFR-2–deleted cells (Figure 3B). Treatment with a neutralizing anti-VEGF antibody reduced the phosphorylated Akt level in VEGFR-1-deleted cells (Figure 3C), suggesting that VEGFR-1 inhibits the activation of Akt by VEGF. To further investigate the relationship between constitutive Akt activation and endothelial cell dysfunction induced by VEGFR-1 deletion, we examined the effect of inhibition of Akt. We infected human endothelial cells with a retroviral vector encoding a dominant-negative form of Akt (DN-Akt)[19] or the empty vector encoding resistance to neomycin alone (Mock). Both cell populations were then infected with shNega or shVEGFR-1. We found that VEGFR-1 deletion markedly inhibited the proliferation of mock-infected endothelial cells (Figure 3D, Mock), whereas this inhibitory effect was significantly ameliorated in DN-Akt–infected cells (Figure 3D, DN-Akt). Consequently, VEGFR-1 deletion significantly impaired tube formation by mock-infected cells, but not DN-Akt-infected cells (Figure 3E). Likewise, inhibition of Akt activation prevented the induction of p21 expression by VEGFR-1 deletion (supplemental Figure II). These results suggest that VEGFR-1 deletion causes dysregulation of activation of the VEGFR-2/Akt signaling pathway by VEGF-A, and that constitutive activation of Akt is related to the impaired ability of VEGFR-1–deleted endothelial cells to proliferate and form capillary-like structures. VEGF-induced phosphorylation of eNOS was enhanced, but production of cGMP was significantly reduced by VEGFR-1 deletion, presumably because constitutive activation of Akt increases cellular reactive oxygen species[19] that inactivate this enzyme (supplemental Figure IC and ID).