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SENESCENCE AND ITS BYPASS IN THE VASCULAR ENDOTHELIUM

Deborah A. Freedman

Vascular Biology Program, Department of Surgery, Children's Hospital, Harvard Medical School, 1 Blackfan Circle, Boston, MA 02115

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1. ABSTRACT

Vascular endothelial cells line the interior of blood vessels. As in other cell types, the proliferative lifespan of endothelial cells is limited; after a given number of replication cycles, they undergo senescence. Angiogenesis, the formation of new capillaries from pre-existing vasculature, is a process that involves endothelial cell proliferation. Angiogenesis thus has the possibility to be limited by the occurrence of senescence in the endothelial cell population. While there is evidence that endothelial cells undergo senescence *in vivo*, there are also data implying that endothelial senescence can be delayed or prevented in certain situations. Such a prevention of senescence would allow continued endothelial cell proliferation and continued angiogenesis in both physiological and pathological settings. This review discusses endothelial cell senescence and its bypass *in vitro* and *in vivo*.

2. INTRODUCTION: SENESCENCE AND IMMORTALIZATION

The proliferative lifespan of cells is limited. After a certain number of replication cycles, cells enter a phase of growth arrest called senescence that is thought to be irreversible (1). There are at least two common pathways that control this arrest. The first is the shortening of telomeres, the sequences of DNA at the ends of linear chromosomes. Telomeres become shorter each time the DNA is copied due to the inability of DNA polymerase to replicate the very ends of the chromosomes (2-5). As telomeres become critically short, their T-loop structure (6) is thought to unfold. Open telomeres likely serve as a signal to the p53 tumor suppressor protein, which can trigger the growth arrest of senescence (7, 8). Senescence induced by p53 likely involves the induction of $p21^{Cip1/Waf1}$, an inhibitor of cyclin dependent kinase (CDK) 2 activity, and may also involve $p21^{Cip1/Waf1}$-independent functions (9, 10).

The other trigger of senescence is the accumulation of $p16^{INK4a}$, an inhibitor of CDK4 and CDK6 (11, 12). The levels of this protein progressively increase as cells undergo cycles of cell division. This increase in $p16^{INK4a}$ occurs independently of telomere shortening (13, 14) until the levels are high enough to induce cell cycle arrest through the retinoblastoma (pRb) pathway (11, 12). Figure 1 contains a schematic diagram of these and other pathways that can contribute to senescence. The relevance of each protein in endothelial senescence is discussed in section 3 below.

Many cell types require both the telomere and the $p16^{INK4a}$ pathways to be bypassed in order for cells to completely circumvent senescence and become immortal. The enforced expression of telomerase (hTert), the reverse transcriptase that extends telomeric sequence, often leads to the immortalization of human somatic cells (15). In human keratinocytes, mammary epithelial cells, prostate epithelium, and some fibroblasts, the immortalized cells have lost expression of the $p16^{INK4a}$ protein (13, 16-19). These observations demonstrate that both telomere attrition and $p16^{INK4a}$ accumulation contribute to senescence in these cell types.

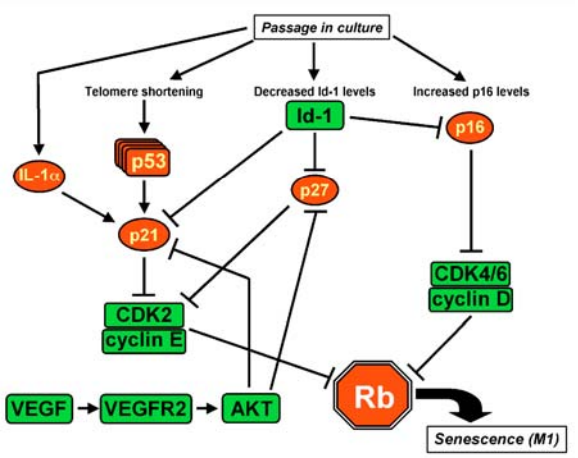

Figure 1. A schematic diagram of the pathways relevant to endothelial senescence. Proteins that promote cell proliferation are shown in green, while those that inhibit proliferation are red. Passage of endothelial cells in culture leads to telomere shortening, increased levels of $p16^{INK4a}$, decreased levels of Id-1, and increased levels of IL-1alpha. Each of these can contribute to an inhibition of CDK-dependent phosphorylation of pRb. Hypo-phosphorylated pRb binds to E2F and prevents the transcriptional activation of genes required for the progression of cells in S phase.

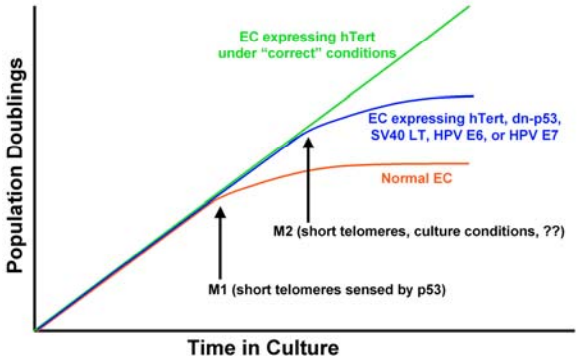

Figure 2. A schematic model of the two phases of endothelial senescence. Mortality checkpoint 1 (M1) is caused by telomere attrition in a p53-dependent manner. Mortality checkpoint 2 (M2), or M2 senescence, is affected by short telomeres and unknown culture conditions. Endothelial cells with stable telomeres can bypass both M1 and M2 and become immortal under the proper conditions.

In other cell types, hTert-induced telomere stabilization can lead to immortalization without the loss of $p16^{INK4a}$. These cells include mesothelial cells, T cells, some fibroblasts, and endothelial cells (13, 14, 20-22). In most of these reports, the mechanism by which these immortal cells tolerate $p16^{INK4a}$ has not been established. Results from our endothelial cell studies indicate that CDK4 activity can remain high in the presence of $p16^{INK4a}$ (20). However, this CDK4 activity is not sufficient for continued cell proliferation (20). These data suggest that $p16^{INK4a}$ does not play a critical role in the induction of senescence in some cell types.

Senescent cells differ from their younger proliferating counterparts in many ways, including in gene expression and morphology (23-25). By careful study of the changes that occur *in vitro* during senescence, it is becoming more and more evident that some of these changes can be observed in many cell types *in vivo* (26). In addition, some observations suggest that cells can circumvent senescence under certain circumstances *in vivo*. Such an event could potentially allow cells to extend their proliferative lifespan and/or to protect them from the deleterious effects of senescence. In particular, there are data indicating that endothelial cells can senesce *in vivo* and in certain situations may circumvent senescence. This evidence is reviewed here, and the implications of the existence of senescent or post-senescent endothelial cells *in vivo* are discussed.

3. THE REGULATION OF ENDOTHELIAL SENESCENCE IN CULTURE

The stabilization of telomeres is required for the immortalization of endothelial cells (20, 27-31). It is not always sufficient for endothelial immortalization, however (20, 27, 32-34). Thus there are certainly other contributing factors to endothelial senescence. A significant lifespan extension without immortalization can be achieved in cultured endothelial cells through various means. There accordingly appears to be at least two points of control of endothelial lifespan, similar to that seen in other cell types (35, 36). The first is typically called senescence or mortality checkpoint 1 (M1); the second is referred to as mortality checkpoint 2 or M2 senescence (Figure 2). The evidence for these two phases of endothelial cell arrest and the factors controlling each are discussed below.

3.1. Telomere shortening, p53, and pRb are critical for M1 senescence

Telomere attrition triggers M1 senescence of human endothelial cells, as evidenced by the fact that telomerase (hTert) expression leads to the bypass of M1 in endothelial cells. Expression of hTert can be sufficient to immortalize a variety of endothelial cell types in culture (27-30). However in many cases, the expression of hTert does not lead to endothelial immortalization. In these cases, the lifespan of the cells is extended to an M2-like arrest (20, 27, 32, 34).

An understanding of the M1 senescence pathway downstream of telomere shortening can be achieved by the study of telomerase-independent interference of M1. The lifespan of endothelial cells in culture can in fact be extended past M1 without hTert expression. This temporary bypass of senescence is often achieved using viral oncogenes that block the activity of p53 and/or pRb. Expression of SV40 virus large T antigen, which inhibits both p53 and pRb, in human bone marrow-derived endothelial cells or human microvascular endothelial cells (HMVEC) derived from mammary tissue led to a significant lifespan extension, but the cells eventually

succumbed to M2 senescence (27, 32). Furthermore, the expression of a dominant-negative p53 construct (37, 38) caused a similar temporary lifespan extension in human umbilical vein endothelial cells (HUVEC) (20). Similarly, expression of either human papilloma virus (HPV)-type 16 E6 or E7, which block p53 and pRb, respectively, extended the lifespan of HUVEC past M1 senescence (39). Moreover, the co-expression of E6 and E7 led to endothelial cell immortalization (39, 40). In these immortal clones, endogenous telomerase activity was induced (39). Taken together, these data support a model in which endothelial senescence at M1 occurs when p53 senses critical telomere shortening and causes a pRb-dependent cell cycle arrest, likely through induction of $p21^{Cip1/Waf1}$. Although this M1 arrest can be bypassed, the continued shortening of telomeres in the absence of hTert expression eventually triggers a second stage of arrest at M2 (20, 27).

3.2. Id-1 represses CDK inhibitors and blocks M1 senescence

Inhibitor of differentiation (Id)-1 is a transcriptional repressor whose expression decreases in cultured endothelial cells over successive passages (41). One gene whose expression is known to be inhibited by Id-1 is $p16^{INK4a}$ (42). In addition, ectopic expression of Id-1 in HMVEC or HUVEC caused a decrease in the expression of the CDK inhibitors $p27^{Kip1}$, $p21^{Cip1/Waf1}$, and $p16^{INK4a}$ in late passage cells. This Id-1 expression subsequently led to an extension of the lifespan of the cells past M1 senescence (41). The telomeres in these cells continued to shorten over time, and although they maintained expression of the endothelial marker CD31, their morphology changed from typical endothelial cobblestone appearance to a more elongated spindle shape. Eventually, the levels of $p16^{INK4a}$ rose, although those of $p21^{Cip1/Waf1}$ and $p27^{Kip1}$ did not. The cells then underwent an M2-like arrest (41). These data indicate that, in addition to the telomere-p53-based induction of senescence, Id-1 loss can contribute to the arrest of endothelial cells at M1, likely through the induction of $p16^{INK4a}$ and other cell cycle inhibitors upstream of pRb. Of note is that Id-1 also leads to transcriptional repression of the endothelial inhibitor thrombospondin-1 (tsp-1) (43), which therefore may be induced in late passage endothelial cells and may also contribute to senescence at M1.

In mouse mammary tumors, expression of the Id genes Id-1 and Id-3 is restricted to the endothelium (44). Mice with deficiencies in these two genes (Id-1 +/-; Id-3 -/-) are viable but have decreased tumor growth and tumor angiogenesis (45). The decreased Id-1 levels in the tumor endothelial cells of these mice is expected to lead to increased levels of $p16^{INK4a}$ and tsp-1 and very possibly also to increases in $p21^{Cip1/Waf1}$and $p27^{Kip1}$, all of which could contribute to the observed decrease in angiogenic potential of these mice.

3.3. Growth factors and cytokines contribute to senescence prevention

Others have reported the extension of endothelial cell lifespan by simple changes in growth medium (46, 47). For example, the addition of VEGF in culture media led to as many as 20 extra population doublings in human dermal HMVEC (47). The levels of the CDK inhibitors $p16^{INK4a}$, $p21^{Cip1/Waf1}$, and $p27^{Kip1}$ were all elevated at the senescence that occurred in the absence of VEGF, and their expression was lowered with the addition of VEGF in the media (47). This relief of CDK inhibition likely results at least in part from AKT activation, which leads to $p27^{Kip1}$ degradation and $p21^{Cip1/Waf1}$ inactivation (48) thus allowing the cells to progress past M1 senescence. However, our results indicate that the inclusion of VEGF in growth media does not affect senescence in HUVEC, which are derived from large vessels (20), suggesting that the role of AKT in the regulation of senescence can differ between various endothelial cell types.

Other pathways that contribute to endothelial senescence may include the expression of cytokines. Interleukin-1alpha (IL-1alpha) mRNA and protein are overexpressed in senescent HUVEC cultures, but not in senescent human diploid fibroblasts (49, 50). In fact, an IL-1alpha antisense oligomer significantly extended HUVEC lifespan without immortalization (50). Furthermore, the IL-1alpha-related peptide, IL-1beta can induce $p21^{Cip1/Waf1}$ expression in a p53-independent fashion in fibroblasts (51) and can also induce growth arrest in melanoma cells independently of both p53 and $p21^{Cip1/Waf1}$ (52). The mechanism of this M1 bypass as induced by inhibition of IL-1alpha expression in relation to $p21^{Cip1/Waf1}$- and p53-dependent mechanisms has not been studied.

3.4. The regulation of endothelial M2 senescence is unclear

The data discussed above suggest that M1 senescence in endothelial cells is controlled at the level of telomere attrition through the p53 and pRb pathways and can be modulated by growth factors and cytokines. Little is understood, however, about the regulation of endothelial M2 senescence. The stabilization of telomeres appears to be required for cells to bypass this point and become immortal, but it is certainly not sufficient. Although endothelial cells whose lifespan has been extended by expression of Id-1 eventually arrest at M2 with high levels of $p16^{INK4a}$ (41), the fact that $p16^{INK4a}$ is not lost in endothelial cells that bypass M2 (20) suggests that this protein may not be critical for this arrest. In fact the immortal endothelial cells in our studies continue to proliferate with little to no CDK4 activity, while CDK2 levels and activity correlate with their sustained growth (20). Thus $p21^{Cip1/Waf1}$ and CDK2 are likely to play a more critical role in the induction of endothelial senescence than $p16^{INK4a}$ at both M1 and M2.

3.5. Products of the Kaposi's sarcoma-associated herpesvirus immortalize endothelial cells

A viral protein that does not inhibit p53 or pRb has also been shown to immortalize endothelial cells by an unusual mechanism (31). The expression of Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor (vGPCR) resulted in the immortalization HUVEC (31). Although these immortal cells did not express telomerase, they contained telomeres of stably long lengths. The

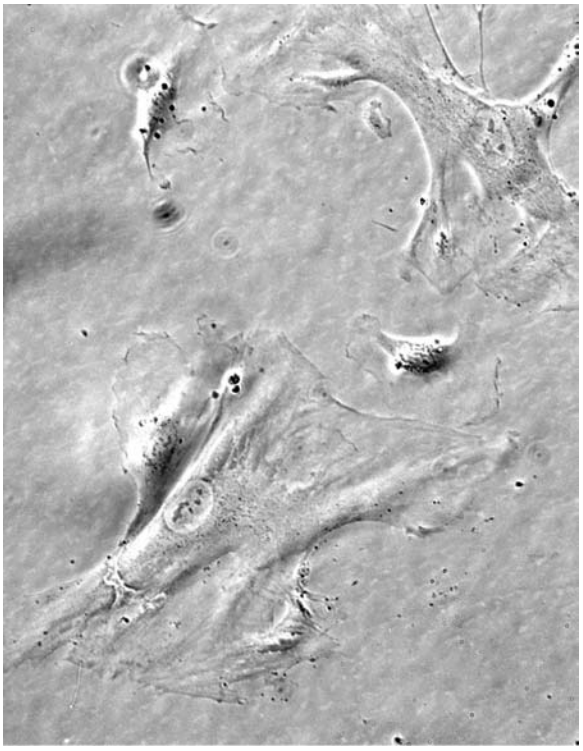

Figure 3. A phase microscope photograph of senescent endothelial cells. Like other cell types, endothelial cells become enlarged and spread flat on the tissue culture plate upon the induction of senescence

telomeres in these cells are most likely maintained by the recombination-based alternative pathway for lengthening of telomeres known as ALT (53, 54). As ALT has not been reported to occur in endothelial cells in any other circumstance, it is possible that expression of vGPCR specifically induces this process. Viral infection with Kaposi's sarcoma-associated herpesvirus causes the transformation of endothelial cells (55) and is thought to be the etiologic agent responsible for the formation of the endothelial tumor, Kaposi's sarcoma (56). ALT could be a contributing factor to the endothelial transformation process. However, human Kaposi's sarcoma cells contain telomerase activity (57), and hTert gene expression can be induced by another KSHV gene, the latency-associated nuclear antigen (LANA) (58). ALT has not been reported in Kaposi's sarcoma cells.

In addition to containing ALT-stabilized telomeres, immortalized HUVEC expressing vGPCR produced ten-fold higher levels of vascular endothelial growth factor (VEGF) than control cells and maintained VEGF receptor 2 (VEGFR2) expression (for review of VEGF signaling see (59)). These proteins formed an autocrine loop that maintained the AKT kinase (also known as protein kinase B or PKB) in its activated state (31). Activated AKT leads to the up-regulation of cyclinD1 and the down-regulation and/or inhibition of CDK inhibitors $p21^{Cip1/Waf1}$ and $p27^{Kip1}$, allowing the cells to progress through the cell cycle (48). Thus the VEGF pathway may also contribute to the bypass of senescence by the expression of vGPCR.

The KSHV LANA protein has also been shown to extend the lifespan of HUVEC without causing their transformation (60). Interestingly, another group demonstrated that LANA can induce the expression of Id-1 protein and that Kaposi's sarcoma tumor cells express high levels of Id-1 protein *in vitro* and *in vivo* (61). Yet another laboratory demonstrated that LANA can induce the expression of hTert (58). It thus appears that KSHV utilizes several mechanisms to bypass senescence of endothelial cells, likely a necessary step in the establishment of Kaposi's sarcoma tumors.

4. CHARACTERISTICS OF SENESCENT AND IMMORTAL ENDOTHELIAL CELLS IN CULTURE

Senescent endothelial cells, like other senescent cell types, have a strikingly modified morphology (Figure 3). The cells become very flat and spread, with an increased cell size. Multinucleated cells often appear in the population. Senescent cells can remain viable and metabolically active though unresponsive to mitogens for several months or more and express senescence-associated beta-galactosidase activity (24, 25).

Gene and protein expression by senescent endothelial cells has been extensively studied (23, 49, 62). Remarkably, each laboratory determined a different set of senescence-regulated genes. Genes and proteins reported to be upregulated in senescent endothelial cells include components or regulators of the extracellular matrix (ECM) such as plasminogen activator inhibitor (PAI)-1, PAI-2, fibronectin, collagen III(1a), and tenascin. Other genes expressed at higher levels in senescent cells include insulin-like growth factor-binding protein (IGFBP)-3, IGFBP-5, IL-1alpha, cyclooxygenase-2, TGF-beta2, acidic fibroblast growth factor (FGF), FGF receptor-activating protein, the growth arrest/DNA damage-inducible protein Gadd153, and prostaglandin-1 synthase. The products of these genes affect the survival, proliferation or physiological responses of endothelial cells or neighboring cells. Intercellular adhesion molecule-1 (ICAM-1) expression is also reportedly upregulated during endothelial senescence and could lead to increased adhesion of other cells to the endothelial cell surface. The expression of other proteins, including connexin 43, endothelial nitric oxide synthase (eNOS), and thymosin beta-10, has been reported to be reduced during endothelial senescence (63-65) and could also effect the matrix or physiology of endothelial cells. Many changes seen in aging vasculature *in vivo*, such as altered ECM content, decreased vasodilatory response, and increased adhesion of monocytes and leukocytes (see below), could be a result of these changes in gene expression.

Endothelial cells that bypass senescence in culture are often aneuploid (28, 30, 39, 66), but can sometimes maintain a seemingly normal karyotype (28, 29). Immortal endothelial cells maintain expression of typical endothelial markers such as CD31, vWF and

alpha(v)beta(3) integrin. They also maintain other characteristics of endothelial cells in culture such as the ability to uptake acetylated low-density lipoprotein (Ac-LDL) and the ability to form tubes on matrigel (28-30). Notably, hTert-immortalized endothelial cells also maintained normal G_1 checkpoint controls (28).

5. AGE-RELATED CHANGES IN VESSELS AND VASCULAR PHYSIOLOGY

Aging humans have increased blood pressure, higher low density lipoprotein (LDL) levels, lower high-density lipoprotein (HDL) levels, and decreased vasodilation. These phenotypic alterations are due to changes in the balance of the endothelial production of growth inhibitors and stimulators and vasodilators and vasoconstrictors. These may include changes in the production and activity of heparin, prostacyclin, nitric oxide, endothelin-1, fibroblast growth factor, transforming growth factor (TGF)-beta, angiotensin II, and IL-1alpha (23). There is also an increase in the adhesion of leukocytes and monocytes to vessel walls, which may partially be explained by increased ICAM-1 expression by senescent endothelial cells (23). Structural changes in aging vessels include thickening of the ECM with altered glycosaminoglycan content and increases in collagen, smooth muscle cell and monocyte content. There is more space evident between the endothelial layer and the internal elastic lamina (67, 68). Vascular endothelial cells become rounder and flatter and have an increased cell volume, reminiscent of senescent endothelial cells in culture (67, 68). These changes in endothelial cell morphology and function contribute to vessel leakiness, to smooth muscle cell (SMC) migration into the subintima, to the recruitment of mononuclear cells into vessel walls, and to the accumulation of collagen (67). These alterations in turn contribute to common vascular pathologies in aged patients, such as atherosclerosis, hypertension, and hypercholesterolemia (67).

Aging humans and other animals also demonstrate decreased ability to undergo angiogenesis (69-71), the formation of new capillaries from pre-existing vasculature. However, this observation, when made in mice, cannot be a function of telomere-based senescence, as mouse telomeres do not shorten. Instead this angiogenic decline appears to result from metabolic changes such as lower nitric oxide release and decrease vasodilation in response to acetylcholine. These changes could be due to organismal age-related changes, but may also be associated with cellular aging of endothelial cells. Factors that contribute to this angiogenic impairment with age may include decreased expression of and response to endothelial growth factors (69, 72-75), decreased endothelial cell migratory ability (76-78), impaired ECM remodeling (76), and an increased tendency towards apoptosis (79). Impaired angiogenesis in older individuals can have important clinical consequences such as impaired recovery from myocardial infarction and impaired wound healing (80-82).

In spite of this decrease in angiogenic function of older endothelial cells and the evidence for endothelial cell replicative aging *in vivo* (see below), human cancer incidence increases as a function of age, suggesting that angiogenesis in tumors is not deficient in aged humans.

6. EVIDENCE OF ENDOTHELIAL SENESCENCE OR ITS PREVENTION *IN VIVO*

The rate of capillary endothelial cell turnover in the mouse retina would require about three years for the entire population to turnover, while it is about 14-times faster in the myocardium (83). Thus although the vasculature is often thought to be quiescent, there is actually continual endothelial cell proliferation in the body. It is therefore quite reasonable to imagine that in the lifetime of a human, endothelial cells may in fact reach senescence. There is, in fact, ample evidence that human endothelial cells do age *in vivo*. For example, telomere shortening and its resultant aneuploidy have been observed in aortic endothelial cells from aged patients (84). In addition, there are observations suggesting that mechanisms exist to prevent or bypass endothelial senescence in situations in which extensive endothelial cell proliferation occurs.

6.1. Atherosclerosis

There is substantial evidence that senescent endothelial cells are present in human atherosclerotic plaques (33, 65, 68, 85). Such cells have been observed based on their increased size and flattened morphology (68) or by their expression of senescence-associated beta-galactosidase activity (33, 65, 85).

Atherosclerosis is a progressive disease in which lipids and fibrous material accumulate inside of arteries. The earliest step in the formation of these plaques is the accumulation of lipoprotein particles in the vessel intima through leaks in the endothelial layer of the vessel wall (86). Next is the adhesion of monocytes to the surface of the endothelium (86). Both of these events could be influenced by the presence of senescent endothelial cells at the site of the lesion. For example, the altered morphology of a senescent cell could damage the integrity of the intercellular contacts and lead to increased vessel permeability. In fact, giant endothelial cells (with a surface area $\geq$ 800 μm^2) have been observed in atherosclerotic lesions in humans (68), consistent with the presence of senescent cells in plaques. The increased expression of ICAM-1 in senescent endothelium (23) could lead to the increased adhesion of monocytes to the endothelial surface. These cells become macrophages, accumulate lipid, and differentiate into the foam cells of the fatty streak that initiate plaque growth (86).

Although the role of endothelial cell senescence in the initiation of atherosclerotic plaque formation is not completely clear, there is substantial endothelial cell proliferation during the growth of the lesion that could result endothelial senescence. The disturbed flow at the site of the lesion leads to endothelial injury, and endothelial cell proliferation occurs during the repair response (86, 87). In addition, there is extensive angiogenesis as capillaries that feed the plaque grow into the intima from the media

(88-90). In fact, as many as 43% of plaque endothelial cells can be undergoing proliferation at a time (91). As this neovascularization enhances plaque growth (88, 92), endothelial senescence has the potential to have both positive and negative effects on atherosclerotic lesions.

6.2. Wound healing

Angiogenesis and extensive endothelial proliferation are major components of the process of wound healing (93). In fact, inhibition of angiogenesis resulted in decreased healing of cutaneous wounds in mice (94). Interestingly, transient telomerase up-regulation has been observed in the proliferating endothelial cells at the tip of healing wounds (95). This observation suggests that human endothelial cells may approach senescence *in vivo* yet have evolved mechanisms to avoid or delay it. Such a system would allow the endothelial cells to proliferate repetitively in response to a wound without the deleterious effects of shortened telomeres (the development of chromosomal abnormalities) and of senescence itself (the inability to proliferate). However, wound healing is in fact impaired in aged animals (81), suggesting that the transient expression of telomerase serves to slow but not halt the cellular aging process. A similar induction of telomerase activity has been observed in several other proliferating human tissues including mitotically active segments of hair follicles (96), the lower third of the intestinal crypt (97), and the endometrium during its proliferative phase (98).

6.3. Cancer

Tumor growth is angiogenesis-dependent (99). Tumor endothelial cells undergo vast proliferation during tumor expansion and the re-growth of tumors that occurs following radiation or chemotherapy. It is therefore evident that human tumor endothelial cells undergo significant proliferative aging. However, human cancer incidence increases as a function of age, and tumor growth does not appear to be slower in older versus younger patients. These observations suggest that tumor endothelial cells may have mechanisms by which to delay or prevent senescence.

There is recent evidence that tumors may in fact contain endothelial cells that have bypassed senescence. *In situ* hybridization studies showed that tumor endothelial cells of many human brain tumors express hTert mRNA (19/19 glioblastoma multiforme; 9/16 anaplastic astrocytomas, and 5/17 low-grade astrocytomas), while normal endothelial cells did not express hTert, as expected (100). This group further demonstrated that a diffusible factor from glioblastoma cells in culture can induce the expression of hTert and telomerase activity in normal HUVEC (101). Consistent with these results, tumor endothelial cells isolated and cultured from renal carcinomas did not undergo senescence but instead proliferated in culture for greater than 50 passages (102). It thus appears that tumors may in fact contain immortal endothelial cells, which could support the sustained growth of the tumor.

There is also limited mouse data that supports this hypothesis. Mouse telomeres are much longer than humans, and most murine somatic cells, unlike those of humans, constitutively express telomerase. Genetically modified mice that lack functional telomerase and that have very short telomeres (103) have in fact been shown to exhibit decreased growth and angiogenesis of explanted subcutaneous tumors (104). However, spontaneous and explanted tumors do in fact grow in mice with short telomeres (104-107). Taken together, these results suggest that although tumor endothelial cells may approach senescence, they may have mechanisms to prevent it. In the case of the telomerase-null mice, the mechanism must involve pathways other than telomerase activation.

7. CONCLUSIONS

The identification of immortal or extended-lifespan endothelial cells in various *in vivo* settings is complicated by the possible recruitment of endothelial precursor cells (EPC) to sites of endothelial cell proliferation. It is possible that the endothelial cells expressing hTert *in vivo* or showing extended lifespans when cultured *ex vivo* could be derived from the stem cell compartment as opposed to the local environment. Although low levels of telomerase activity have been observed in EPC, they undergo senescence after only a few passages in culture (108-110). The degree that these cells contribute to the angiogenesis of wound healing, tumor growth, atherosclerosis, and other processes remains to be definitively established (111-114).

One important implication of endothelial cell aging *in vivo* is especially evident in the growth and treatment of tumors. If telomeres shorten in tumor endothelial cells, chromosomal abnormalities can arise from the end-to-end joining of chromosomes and the subsequent process of fusion-bridge breakage (107, 115). None of the groups that isolated endothelial cells from tumors analyzed the karyotype of the cells (102, 116, 117). Such genomic instability in tumor endothelial cells could lead to the development of resistance to anti-angiogenic drugs by these cells, which have been assumed to be genetically stable targets. It is clear that tumor endothelial cells have very different gene expression profiles than normal endothelial cells (118, 119), which may indicate that they are not stable genetically.

There are many *in vivo* situations in which mature endothelial cells proliferate, and they therefore have the potential to reach or bypass senescence. Angiogenesis is involved in physiological processes such as the female reproductive cycle, inflammation, and wound healing (120). In addition, pathological angiogenesis occurs in many diseases, including cancer, atherosclerosis, and diabetic retinopathy (121). Angiogenesis may also play a positive role in the revascularization of ischemic heart and other organs (122, 123) and in the engraftment of transplanted tissues and engineered biopolymers (124). A better understanding of the regulation of endothelial cell lifespan will aid in the development of future therapies and the identification of novel targets for the therapeutic intervention of endothelial senescence and its bypass.

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Send Correspondence to: Dr Deborah Freedman, Vascular Biology Program, Children's Hospital, Karp Family Research Laboratories, Floor 12, 1 Blackfan Circle, Boston, MA 02115, Tel: 617-919-2402, Fax: 617-730-0231, E-mail: Deborah.freedman@childrens.harvard.edu

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