Review

Vascular smooth muscle cell senescence in atherosclerosis

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Abstract

Markers of cell senescence have been identified in both the blood and vessel wall of patients with atherosclerosis. In particular, vascular smooth muscle cells (VSMCs) derived from human plaques show numerous features of senescence both in culture and in vivo. This review summarises the evidence for VSMC senescence in atherosclerosis, and outlines the mechanisms and triggers leading to their senescence.

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1. Introduction

The most serious complications of atherosclerosis arise from the thrombotic occlusion of arteries consecutive to the erosion or rupture of an atherosclerotic plaque. These manifestations are directly associated with acute coronary syndromes, myocardial infarction and stroke. The incidence of plaque rupture and thrombotic events is largely correlated with specific morphological and histological features described for unstable plaques [1]. In particular, vulnerable lesions have a thin fibrous cap overlying a large core of oxidized lipids and infiltrated inflammatory cells. In approximately 60% of cases, it is the fibrous cap of an atherosclerotic plaque that ruptures [2]. The structural components of atherosclerotic plaque caps consist primarily of VSMC-derived collagen, elastin, proteoglycans and extracellular matrix (ECM). In order to maintain stability, plaque caps require greater collagen and ECM content than the adjacent intima [3]. As the cap ages, it thins, and becomes more inclined to rupture. Fibrous caps of unstable plaque contain less collagen and ECM components and fewer VSMCs than caps from stable and more intact plaques [3].

Although plaque rupture and erosion can induce vessel occlusion, a large proportion of ruptured plaques are clinically silent. For example, plaques demonstrate multiple sites of healed rupture [4], suggesting that the majority of disrupted plaques are repaired. Repair is due to a combination of VSMC invasion of thrombus, proliferation and matrix synthesis. The repair site is gradually remodeled over time, with alterations in matrix components, and resorption of thrombus. As plaque rupture and repair is also associated with plaque growth [4], the efficiency of the repair process critically determines whether the plaque undergoes subsequent rupture or causes lumen narrowing.

VSMC proliferation is central to the repair process. Release of growth factors such as the basic fibroblast growth factor (bFGF), insulin-like growth factor (IGF) and platelet-derived growth factor (PDGF) by inflammatory cells, stimulates VSMC proliferation observed in the vasculature (reviewed in Refs. [5,6]). Proliferation of VSMCs occurs with increased frequency in early lesions, in contrast only low levels of proliferation (<1% of all cells) are seen in advanced atherosclerotic plaques [7–9]. This low level of cell proliferation, even after plaque rupture, and despite the presence of mitogens released within the plaque, is the first suggestion that VSMCs in the plaque might be senescent. Senescence of VSMCs in the fibrous cap may contribute significantly to inefficient plaque repair, with subsequent plaque instability. Senescent cells also overexpress genes that directly promote plaque instability including adhesion molecules (ICAM-1 [10]), regulators of haemostasis (PAI-1...
and matrix metalloproteinases (collagenase and stromelysin [12]). The recognition that VSMC senescence may be important to the behaviour of advanced plaques has resulted in studies designed to examine evidence for plaque VSMC senescence, and the mechanisms underlying senescence.

2. Evidence of senescence of VSMCs in plaques

Plaque VSMCs have been isolated from human endarterectomy specimens, subcultured in vitro, and their properties compared to those of VSMCs derived from the media of normal arteries [13–16]. Plaque VSMCs have an enlarged, flattened and stellar shape (Fig. 1) with high amounts of cytoplasmic vacuoles and lysosomes. These are features of normal VSMCs undergoing replicative senescence in culture [17]. Cellular senescence can be defined as cell cycle arrest accompanying the exhaustion of replicative potential [18]. Unlike quiescence, senescence is irreversible, and cells cannot initiate cell cycle transit in response to mitogens. Besides their characteristic morphology, senescent cells display undetectable rates of cell proliferation and characteristic patterns of gene expression, including markers such as SAβG [19] and cell cycle regulators [20–22]. Importantly, cells undergoing replicative arrest have short telomeres, the structures comprising the ends of chromosomes. As reported below, there is accumulating evidence demonstrating that some of these features of senescent cells can be observed in plaque cells.

2.1. Cell proliferation

A striking characteristic of plaque VSMCs in vitro is their restricted capability to proliferate [13–15,23,24]. Even though this feature is not universally found [25], isolated plaque-derived VSMCs show lower rates of cell proliferation, and lower % of cells in S phase of the cell cycle [15,23]. In addition, plaque VSMCs undergo premature senescence. Normal VSMCs may be cultured for 10–20 passages under routine conditions, depending in part upon the age of the donor. In contrast, plaque VSMCs rarely remain dividing beyond 5–10 passages [15,23,24]. Whilst the life span of both plaque and normal VSMCs can be extended, in some cases to become immortalized [23,24], the extended life span of plaque VSMCs is still less than normal VSMCs with the same genetic manipulation, and the rate of immortalisation of plaque VSMCs is lower than normal VSMCs. These studies reinforce the concept that plaque VSMCs are pre-senescent.

2.2. SAβG expression

Although senescent cells have very distinctive profiles of gene expression when compared to highly proliferating cells at early passage, finding specific markers that discriminate between senescent and non-proliferating quiescent or terminally differentiated cells has been more challenging. The activity of the senescence-associated β-galactosidase (SAβG) has been described as a marker of senescent cells in vitro and in vivo. β-galactosidase is a metabolic enzyme highly expressed in pre-senescent and senescent cells [26]. SAβG activity corresponds to β-galactosidase activity measured in pH conditions where only high levels of the enzyme are detectable, and proportionally correlate with lysosomal content [27]. Plaque VSMCs isolated from human endarterectomy samples not only possess a large and flattened shape, but also display high SAβG staining even at early stages of culture (Fig. 1).

SAβG-positive cells are detected in both the endothelium and intimal VSMCs of advanced human atherosclerotic plaques, but not in the vessel media [28,29], suggesting that vessel wall senescence may be a feature of advanced plaques. SAβG staining is also found in VSMCs of the neointima of rabbit carotid arteries injured by repeated endothelial denudation [30]. However, within the plaque, many cells showing SAβG staining are macrophages due to their high lysosomal content, and double labeling is required to identify the origin of SAβG-positive cells. In particular,
there is extensive evidence that endothelial cell senescence contributes to atherogenesis (reviewed in Refs. [31–36]).

2.3. Cell cycle regulators

VSMCs isolated from human plaques display significantly reduced percentage of cells in S phase of the cell cycle, a higher percentage of cells in G1, and a longer mean mitotic time compared with VSMCs isolated from normal vessels [37]. Since cell cycle regulators controlling the G1/S transition have a specific profile of expression in senescent cells, several studies have explored the pattern of expression of these specific genes in order to appreciate the proliferative status of VSMCs in pathological conditions.

The G1/S transition is primarily regulated by the retinoblastoma protein pRB, product of the rb tumor suppressor gene. pRB exerts its negative regulation on the cell cycle through binding of E2F transcription factors E2F-1, E2F-2 and E2F-3, rendering them ineffective as transcription factors [38]. The pRB/E2F complex is dissociated in early G1 by phosphorylation of pRB by the cyclin-dependent kinases (CDKs) 2, 4 and 6. E2F becomes then available to activate the subset of genes required for S phase entry. While these kinases are constitutively expressed in cells, their activity depends on the expression of their respective activators, cyclin D1, D2 and D3 for CDK4 and CDK6 and cyclin E for CDK2. As for fibroblasts, growth factors or serum-stimulated non-confluent VSMCs show increased pRB phosphorylation, increased expression of cyclin D and E and the induction of E2F-dependent S phase genes [39,40]. Plaque VSMCs display an opposite pattern of pRB phosphorylation [40], which may explain their reduced E2F transcriptional activity (Fig. 2).

The cell cycle is tightly regulated by multiple checkpoints, which allow the cell to verify the integrity of its DNA. If cell damage is detected, a series of cyclin-dependent kinase inhibitors, including p16ink4, p21cip1 or p27kip1 proteins, are expressed at high levels and directly or indirectly block the activity of the CDKs involved in G1. Indeed, overexpression of p21 and p27 will force VSMCs into G1 growth arrest [41]. When compared to control cells, plaque VSMCs have increased expression of p16 and p21, a reduction in pRB phosphorylation, and increased levels of E2F-1/pRB complex [40]. In vivo, p27kip1 expression is elevated in non-proliferating cells from normal and diseased vessels. In contrast, p21cip1 is elevated only in the atherosclerotic plaque [40,42] and co-localizes with elevated p53 [43], suggesting that the p21 transactivation may be dependent on p53 stabilization.

Confirmation of the role of these proteins in VSMC senescence comes from studies with single or double inactivation of p53, pRB or both. The life span of normal human VSMCs can be extended by inactivation of p53 or pRB alone, although inactivation of both p53 and pRB is more efficient and is required for full immortalisation [23,24]. In contrast, inactivation of p53 does not extend the life span of plaque VSMCs, and inactivation of pRB alone induces apoptosis in these cells [23]. Inactivation of both tumour suppressor genes is required for extension of life span of plaque VSMCs, and even then, immortalisation is rarely achieved [23,24]. Whilst this confirms that cell cycle arrest mediated via p53 and pRB are important for senescence of VSMCs, it implies that plaque VSMCs need inactivation of multiple checkpoints to bypass senescence.

2.4. Telomere length

Telomeres are DNA-protein complexes at the end of eukaryotic chromosomes that protect them from degradation, recombination or fusion [44]. Telomeres consist of double-stranded repeats of the AATGGG sequence and are ended by a single stranded 3’ overhang folded in a structure referred as the T loop (Fig. 3). Conventional polymerases need primers to elongate linear DNA, therefore cannot support the replication of the very end of telomeres [45]. Telomeres are maintained by the enzyme telomerase, which contains an intrinsic RNA subunit (hTERC) and serves as a template for its catalytic subunit (hTERT) [46]. During division of somatic cells, low levels of telomerase do not allow full replication of telomere ends, resulting in progressive shortening with each division [47,48]. As telomere shortening is an irreversible feature, the mean telomere length of a cell population can therefore be taken as an indicator of its replicative history [49].

Recent studies have identified an inverse relationship between peripheral leukocyte telomere length and either
Fig. 3: Structure of human telomeres. The extremity of mammalian chromosomes is composed of hexanucleotide repeats that are protected from degradation by a very specific DNA/protein structure. The overhang, a 3’-single stranded sequence, folds back into the double stranded sequence of the telomere, creating the displacement loop (D-loop), and resulting in a super-structure called the T loop. The T loop is capped by telomere-associated proteins, which stabilize the structure or regulate telomere length. These include the telomere-repeat binding factors TRF1 and TRF2; the TRF1-interacting nuclear protein (TIN2) and its partner PIP1; DNA repair factors, such as the MRE11 complex and the DNA-dependent protein kinase (DNA-PK) complex; a variety of proteins and enzymes that interact with and regulate TRF1, such as tankyrases (TANK1, TANK2). It has been proposed that dissociation of TRF2 from telomeric DNA disrupts the T loop, an event that by itself can cause the loss of the overhang and trigger the DNA damage response and senescence.

atherosclerosis or premature myocardial infarction [50–53]. Telomere length in leukocytes is also negatively associated with mortality due to cardiovascular disease [54]. In these studies, the average leukocyte telomere length in patients with severe coronary artery disease either before or after myocardial infarction was 300 bp shorter than those from the control groups [50,51], corresponding to an equivalent difference of 11 years of age [51]. Despite this difference, the rate of decrease in telomere length per year was similar in both groups. Several explanations have been proposed to support this observation. First, the telomere length of leukocytes, which is genetically determined at birth, may predispose to age-related diseases. In this model, senescent leukocytes contribute to atherosclerosis via an unknown mechanism. Second, inflammation associated with atherosclerosis accelerates leukocyte turnover, such that telomere loss is a marker of leukocyte senescence, but does not per se contribute to atherosclerosis. Inflammation would be both the inducer of telomere loss and predisposes to atherosclerosis, but leukocyte telomere loss per se does not promote atherosclerosis. In support of this, telomeres in T lymphocytes from patients with chronic inflammatory disease are significantly shorter than in the control group [55]. In addition, it has been proposed that age-related exhaustion of bone marrow vascular progenitor cells may increase the development of atherosclerosis [56]. Although a fraction of intimal VSMCs in primary atherosclerotic plaques have been proposed to derive from circulating blood cells [57], their impact on cellular senescence is unclear. For example, a feature of stem cells is that they have increased replicative potential, and would therefore be predicted to undergo senescence less readily than cells from the vessel wall.

There is also direct evidence of telomere loss in cells of the vessel wall in atherosclerosis. VSMC or endothelial cell telomere size is not uniform throughout the vascular, and differences between the intima and media have been observed [49,58,59]. Vessels subject to high haemodynamic stress have significantly shorter telomeres in their intima when compared to arteries with low haemodynamic stress [58], suggesting that high cell turnover at sites of high stress cause short telomeres. In addition, telomere length in the media and intima of distal and proximal abdominal aorta has been shown to negatively correlate with predisposition to atherosclerosis [60]. The intima showed an age-dependent inverse correlation with age, with higher attrition rates in the distal than the proximal segments. The atherosclerosis grade inversely correlated with telomere length but the correlation was lost after adjustment for age [60]. Whilst these studies suggest that atherosclerosis is associated with telomere loss, they do not prove that telomere loss promotes atherosclerosis rather than just being a marker of increased cell turnover, nor do they identify the mechanism of telomere loss.

3. Mechanisms of cellular senescence

3.1. Telomere shortening and senescence

Replicative senescence may be induced by the reduction of telomere length, changes in structure such as telomeric fusion or dicentrics [61], or loss of telomere-bound factors. After repeated divisions, telomeres may reach a critical length or structure whereby irreversible growth arrest or senescence is triggered [49,62]. In addition, there is substantial evidence of a cause and effect relationship between telomerase expression and manifestations of senescence. Ectopic expression of the catalytic subunit of telomerase (hTERT) can prevent replicative senescence in several cell types such as fibroblasts or epithelial cells, despite the fact that telomeres were always protected [63–67]. hTERT may also influence the interaction of telomeres with the nuclear matrix, increase chromosome stability, decrease telomere fusion, reduce spontaneous chromosome breaks and enhance DNA repair. These effects are independent of the effects of telomerase on telomere length [68]. Telomere uncapping can also cause cell senescence independent of telomere length and telomerase activity. For example, overexpression of a negative mutant of the telomere capping protein TRF2 causes senescence in fibroblasts or fibrosarcoma cells, even though the cells retain their long telomeres [69–71]. These experiments support the idea that as telomeres shorten they lose their protein-binding properties, possibly causing irreversible damage to the T loop structure [69,72].

Although ectopic expression of hTERT can dramatically extend the life span of normal human VSMCs [73], the evidence that hTERT expression or activity directly affects atherosclerosis is controversial. Human VSMCs and ECs
express low levels of hTERT [74,75] and hTERT activity is readily detectable only when cells are proliferating [75]. Indeed, hTERT is a target of E2F-1 transcription factor, where mRNA expression of the enzyme increases when cells enter the S phase [76,77]. Phosphorylation of hTERT in vascular cells, along with its activation and nuclear translocation, offers an additional level of regulation of the enzyme activity at the post-translational level [78,79].

ApoE null mice defective in hTERT show reduced atherosclerosis suggesting that decreased hTERT expression is protective in atherosclerosis [80]. In this study, hTERT was knocked-out in all cells that comprise the plaque, and the effects of telomerase deficiency were linked to the impaired function of inflammatory cells. In contrast, mice in which various aspects of senescence are accelerated show increased atherosclerosis, suggesting that cellular senescence ultimately promotes atherogenesis [81]. It is therefore possible that manipulation of cellular senescence in mice can have variable effects on plaque development depending upon the stage of lesion formation.

Whatever the mechanism of telomere damage, telomere dysfunction elicits a general DNA damage response and growth arrest via the activation of a protein kinase cascade including the kinases ataxia telangiectasia mutated (ATM) and ATM-related kinases (ATR) [82,83]. The DNA damage response involves the phosphorylation and recruitment of the kinases ATM, ATR and DNA protein kinase (DNA-PK) [84] at the site of DNA damage. This results in the phosphorylation of histones such as H2AX, the association of DNA repair enzymes and cofactors (53BP1, MDC1/NFBD1 and NBS1), and activation of the transducer proteins Chk1 and Chk2 [83](For review see Ref. [85]). Targeted damage of either telomeres or genomic DNA finally converge on to a common pathway characterised by the activation of at least one of the cell cycle inhibitors p21<sup>Cip1</sup> (via p53) or p16<sup>INK4a</sup> [86,87] (Fig. 4), responsible for inducing growth arrest and senescence.

Senescence is also characterized by the presence of a greater number of immunochemically detected DNA damage foci. Each focus reveals the presence of the DNA repair complexes at the site of DNA damage [88]. It is not certain, however, whether the DNA damage foci are associated with telomeric DNA. High resolution imaging of senescent fibroblasts show that the foci-associated protein γ-H2A-X was found in the telomeric DNA regions [84] and was co-immunoprecipitated with a sub-telomeric DNA region in a chromosome immunoprecipitation assay [88]. In contrast, other studies found that γ-H2A-X does not colocalize with telomeric DNA or TRF2 proteins [89,90]. Whatever the mechanism, these results support the hypothesis that telomere-mediated DNA damage response is involved in replicative senescence. Nuclear DNA damage foci are present during the cellular senescence, and for this reason the protein components of foci have been proposed as markers of senescence [90]. It will be of particular interest to study these proteins in comparison to current markers of senescence and follow their behaviour in cardiovascular disease.

### 3.2. Oxidative stress and senescence

Cell division is not the only path to senescence. Irreversible growth arrest can also be triggered in response to a variety of stresses, and as such is termed "stress-induced premature senescence" (SIPS). In vitro, SIPS can be elicited by overexpressing Ras and Raf oncogenes [91,92], radiation [93,94], or chemical agents producing any form of DNA damage and oxidant stress [94–97]. Oxidative stress, in
addition to excessive cell proliferation, is one of the most physiologically relevant triggers of cell senescence in pathological conditions. Intracellular generation of superoxide anions, hydrogen peroxide and hydroxyl radicals, can produce DNA strand breaks and various types of DNA bases modifications, including the highly mutagenic oxidation of guanine residues into 7,8 dihydro 8-oxo-guanine (8-oxoG) [98].

Increased levels of ROS are found in atherosclerosis in all layers of the diseased arterial wall, and particularly in the plaque itself [99,100]. Whether generated by the cellular NAD(P)H oxidases, xanthine oxidase, myeloperoxidase or the mitochondrial oxidative metabolism, increased levels of ROS may account for the high levels of DNA lesions in atherosclerotic plaques. Higher levels of 8-oxoG DNA adducts are found in human carotid endarterectomy specimens compared with the normal media, associated with increased expression of DNA repair enzymes, such as DNA-PK and PARP-1 [101]. Evidence of fragmented DNA was also found in plaque macrophages and VSMCs [101]. A significant and reversible increase in DNA damage has also been observed in atherosclerotic plaques from cholesterol-fed rabbits [102], which regresses on withdrawal of high fat feeding. Altogether, these studies directly implicate oxidative DNA damage, DNA repair and cell senescence in atherosclerosis.

In vitro, oxidant stress induced by chronic hydrogen peroxide (H₂O₂) treatment [103], hyperoxic culture conditions [96] or alterations of the cell’s anti-oxidant properties [27] can all accelerate senescence. Treatment of cultured fibroblasts with H₂O₂ can induce telomere single strand breaks that may promote telomere shortening [104] and consequently induce premature cell cycle arrest by triggering pathways converging towards activation of the G₁-associated cell cycle inhibitors (p21CIP1, p16INK4). Increased telomere loss per division can also occur in individual cells due to a telomere-specific deficiency in base excision repair. This mechanism leads to preferential accumulation of ROS-induced single-stranded DNA breaks [105], preventing replication of distal segments of chromosomes when cells divide. Alternatively, repeated stress dramatically increases the proportion of cells undergoing growth arrest [106,107], suggesting that oxidative stress may exert selection pressure with replication of a subset of VSMCs in vivo. Oxidative stress can also induce premature senescence independently of telomere shortening. Pulsed treatments with low doses of H₂O₂ in fibroblasts can cause irreversible cell cycle arrest accompanied by an increase in SAβG staining without concomitant changes in telomere length [108].

Accumulation of oxidative damage to genomic DNA may contribute to SIPS, but also to replicative senescence since senescent cells in vitro have higher levels of 8-oxoG DNA base modifications [109]. These results are consistent with the observation that senescent cells present more DNA damage foci, even in non-telomeric sequences [88]. Therefore, the distinction between replicative and oxidative stress-induced senescence is not clearly delineated, and both pathways lead to SAβG overexpression, even though distinct cell cycle inhibitors may be involved.

4. Conclusions

There is increasing evidence showing that cell senescence contributes to atherosclerosis. Markers of cell senescence are present in peripheral blood leukocytes in patients with atherosclerosis. Human vascular cells, particularly VSMCs, show evidence of senescence both in culture and in plaques. Cell senescence is likely to be due to both replicative senescence of cells forming the plaque, and stress-induced premature senescence, induced for example by oxidative stress. As VSMC senescence may contribute to plaque instability and impair plaque repair after rupture, prevention of cell senescence may become an increasingly important therapeutic target in atherosclerosis.

References

[11] Zeng G, McCue HM, Mastrangelo L, Millis AJ. Endogenous TGF-beta activity is modified during cellular aging: effects on...


