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TOPICAL REVIEW

Ageing induced vascular smooth muscle cell senescence in atherosclerosis

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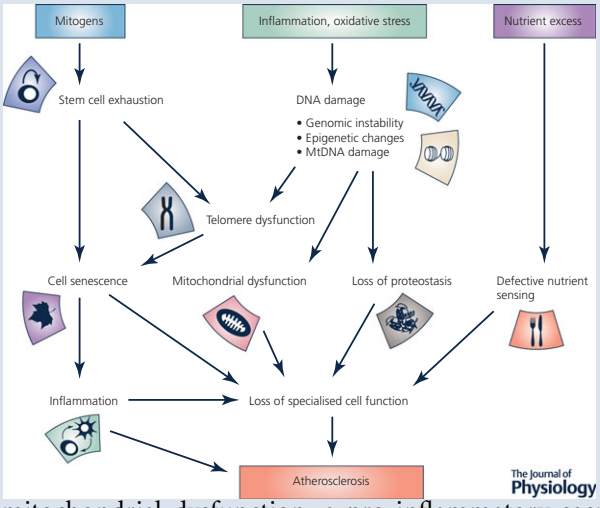

Abstract Atherosclerosis is a disease of ageing in that its incidence and prevalence increase with age. However, atherosclerosis is also associated with biological ageing, manifest by a number of typical hallmarks of ageing in the atherosclerotic plaque. Thus, accelerated biological ageing may be superimposed on the effects of chronological ageing in atherosclerosis. Tissue ageing is seen in all cells that comprise the plaque, but particularly in vascular smooth muscle cells (VSMCs). Hallmarks of ageing include evidence of cell senescence, DNA damage (including telomere attrition), mitochondrial dysfunction, a pro-inflammatory secretory phenotype, defects in proteostasis, epigenetic changes, deregulated nutrient sensing, and exhaustion of progenitor cells. In this model, initial damage to DNA (genomic, telomeric, mitochondrial and epigenetic changes) results in a number of cellular responses (cellular senescence, deregulated nutrient sensing and defects in proteostasis). Ultimately, ongoing damage and attempts at repair by continued proliferation overwhelm reparative capacity, causing loss of specialised cell functions, cell death and inflammation. This review summarises the evidence for accelerated biological ageing in atherosclerosis, the functional consequences of cell ageing on cells comprising the plaque, and the causal role that VSMC senescence plays in atherogenesis.

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Abstract figure legend Pathways and Processes leading to accelerated ageing in atherosclerosis. Adapted from Lopez-Otin C, Blasco MA, Partridge L, Serrano M & Kroemer G. (2013). The hallmarks of aging. *Cell* 153, 1194-1217.

Abbreviations ATM, ataxia telangiectasia mutated; BMC, bone marrow-derived cell; CAD, coronary artery disease; DDR, DNA damage response; EC, endothelial cell; EPC, endothelial progenitor cell; γ-H2AX, phosphorylated form of histone 2A protein X; IGF1, insulin-like growth factor-1; iNOS, inducible nitric oxide synthase; MtDNA, mitochondrial DNA; ROS, reactive oxygen species; SASP, senescence-associated secretory phenotype; SIPS, stress-induced premature senescence; SOD, superoxide dismutase; UPR, unfolded protein response; VSMC, vascular smooth muscle cell.

Introduction

Atherosclerosis is the commonest cause of death in the UK, and is projected to be the commonest cause of death in the world. The risk factors for atherosclerosis are well known, but one of the most important is increasing age. Ageing is associated with increased incidence, prevalence and mortality associated with atherosclerosis. Atherosclerosis is a slowly progressive disease, with histological evidence of plaque formation often as early as the second or third decade of life. Plaques develop over decades, growing both by gradual expansion and by episodes of rupture and repair that lead to rapid plaque growth. The association between chronological ageing and atherosclerosis has been explained by this gradual evolution of plaques through histologically defined stages that ultimately result in advanced lesions that are associated with clinical events. However, more recent evidence suggests that plaques also manifest accelerated biological ageing that is out of proportion to the chronological age. These 'hallmarks of ageing' are seen in advanced lesions, but their earliest features are also present as plaques develop.

Tissue hallmarks of ageing

Although the functional consequences of ageing vary according to the tissue involved, there are a number of common markers or features of ageing that are seen to a variable extent across all tissues. These 'hallmarks of ageing' have been summarised and illustrated in an excellent recent review (Lopez-Otin *et al.* (2013) and Fig. 1). These features include damage to both nuclear and mitochondrial DNA including attrition of telomeres and epigenetic changes, resulting in cellular senescence and exhaustion of stem/progenitor cells. Other consequences of cell senescence include defects in protein processing (proteostasis) and in sensing of nutrients, and also altered intercellular communication, for example by alterations in immune surveillance and release of pro-inflammatory cytokines. We will review the evidence for these 'hallmarks of ageing' in atherosclerosis, with a particular focus on vascular smooth muscle cells (VSMCs).

Genomic instability. DNA damage is evident in cells of human atherosclerotic plaques (particularly VSMCs and macrophages), including strand breaks, telomere shortening and base oxidation, particularly of guanosine residues (8-oxo-G). Both damage and markers of DNA repair increase with disease severity (Martinet *et al.* 2002; Matthews *et al.* 2006; Mahmoudi *et al.* 2008) suggesting that ongoing damage or defective DNA repair is an important feature of atherogenesis. DNA strand breaks and chromosomal damage are present in both plaque VSMCs (Gray *et al.* 2015) and circulating cells of patients with atherosclerosis, suggesting the interplay of both local and systemic factors that promote ageing in parallel with atherosclerosis. For example, DNA damage in circulating cells is associated with disease severity and correlates with a higher micronucleus index (a marker of genetic instability) compared with healthy controls (Botto *et al.* 2001).

Markers of DNA damage develop and may persist during atherosclerosis in animal models of atherosclerosis, and also in culture. For example, 8-oxo-G and DNA strand breaks develop in fat-fed rabbits, associated with widespread expression of DNA repair enzymes; these changes revert on transfer to a normal diet, although 8-oxo-G may persist (Martinet *et al.* 2001). Similarly, increased DNA damage is seen in plaque-derived VSMCs compared with normal VSMCs in culture, evidenced by increased expression of multiple DNA damage markers, including the phosphorylated forms of the ataxia telangiectasia mutated (ATM) and histone 2A protein X proteins (γ-H2AX), and a longer tail length on Comet assay, a marker of DNA strand breaks (Mahmoudi *et al.* 2008; Gray *et al.* 2015). Thus, DNA damage (and by inference its consequences) can persist in vascular disease despite correction of the local extracellular environment.

Telomere attrition. Telomeres are high order structures composed of tandem repeats of the sequence TTAGGG that end in a single-stranded 3′-overhang of 50–300 nucleotides (Aubert & Lansdorp, 2008). This structure folds back to form a 'T-loop' that caps the end of each chromosome (Griffith *et al.* 1999), and whose integrity depends on specific proteins that form the 'shelterin complex'. This protein complex comprises telomeric repeat-binding factor (TRF) 1, TRF2, protection of telomere protein 1 (POT1), TRF1-interacting nuclear factor 2 (TIN2), tripeptidyl-peptidase 1 (TPP1) and

telomeric repeat-binding factor 2-interacting protein 1 (RAP1). Telomere protection depends particularly upon TRF2 and POT1 (van Steensel *et al.* 1998; Karlseder *et al.* 1999; Wu *et al.* 2006), which allow cells to distinguish between random DNA breaks and the natural end of the chromosome (Aubert & Lansdorp, 2008). Repeated cell division (d'Adda di Fagagna *et al.* 2003) or loss of key telomere binding proteins (Karlseder *et al.* 1999) destabilizes telomere integrity, which induces a DNA damage response (DDR) and leads to cellular senescence.

The telomeres of both VSMCs (Matthews *et al.* 2006) and ECs (Ogami *et al.* 2004) are shorter in atherosclerotic plaques relative to the normal vessel wall. Shorter telomeres are also seen in circulating endothelial progenitor cells (EPCs) (Carracedo *et al.* 2011), and in leukocytes in patients with atherosclerosis compared to control subjects (Brouilette *et al.* 2003, 2007), where they are inversely correlated to cardiovascular disease risks in patients with subclinical disease (Panayiotou *et al.* 2010; Willeit *et al.* 2010). Although it is hard to demonstrate critically short telomeres in these studies, ectopic activity of telomerase (the enzyme that maintains telomere length) can dramatically increase lifespan of both plaque and normal VSMCs (Matthews *et al.* 2006), suggesting that short telomeres and low levels of telomerase expression/activity are functionally important in VSMC senescence. In addition, whilst telomere length may reflect previous replication, arterial segments resistant to atherosclerosis, such as internal mammary artery or ascending aorta, have longer telomeres than the aortic regions prone to the disease (Nzietchueng *et al.* 2011; Chang & Harley, 1995). This difference is age-independent, suggesting the existence of intrinsic genetic or developmental variations in telomere regulation may underlie location-specific predisposition in atherogenesis.

Epigenetic modifications. Epigenetics defines genetic changes that are not related to differences in the coding sequences. For example, changes in DNA methylation and histone modification of vascular genes and growth factors are observed in the development and progression of atherosclerosis (Schiano *et al.* 2015). DNA hypomethylation occurs in monocytes, VSMCs and plaques of patients with atherosclerosis (Pogribny & Beland, 2009), while additional studies using ApoE$^{-/-}$ mice have shown that DNA hypomethylation represents a significant risk factor associated with susceptibility to atherosclerosis (Lund *et al.* 2004). The global DNA hypomethylation that predominates in human atherosclerosis causes activation of a number of gene clusters (Wang *et al.* 2014; Aavik *et al.* 2015).

Epigenetic changes are most likely due to changes exerted by DNA damaging agents. For example, DNA

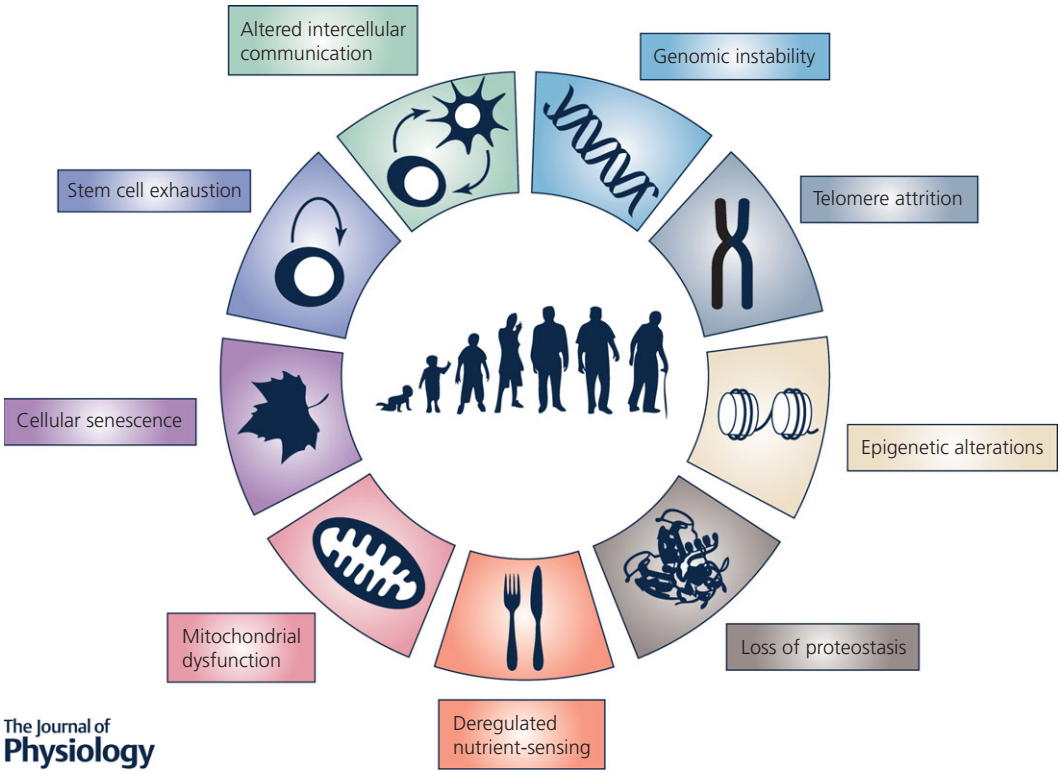

Figure 1. Hallmarks of tissue ageing
A full explanation is provided in the text. Adapted from Lopez-Otin *et al.* (2013).

damage in vascular cells can result from exposure to oxidative stress, and under normal circumstances there are a variety of antioxidant enzymes that remove these damaging reactive oxygen species from the environment. Recent studies have shown that inflammatory cytokines can alter the expression of mediators of oxidative stress such as inducible nitric oxide synthase (iNOS) by causing changes to the chromatin structure of the promoter (Chan *et al.* 2005), and DNA hypomethylation of the gene for the antioxidant enzyme superoxide dismutase (SOD) results in reduced expression (Laukkanen *et al.* 1999). These gene expression changes are likely to force the cellular redox balance towards that of a highly oxidising environment that may potentially increase cellular DNA damage and enhance the progression of atherosclerotic lesions.

Loss of proteostasis. Proteostasis describes the biological pathways within cells that control the biogenesis, folding, trafficking and degradation of proteins present within and outside the cell. Protein storage defects, misfolding or increases in protein secretion can cause endoplasmic reticulum (ER) stress, which in turn induces activation of a series of ER-resident proteins, including activating transcription factor-6 (ATF-6), inositol requiring protein-1 (IRP-1), and protein kinase RNA-like ER kinase (PERK). These proteins direct signalling pathways that relieve ER stress in a process known as the unfolded protein response (UPR). Overall protein ubiquitination in atherosclerotic plaques is significantly increased, with an increase in proteasome activity in early atherosclerosis, but a decrease in advanced atherosclerosis (Wang *et al.* 2014). Proteasome inhibition can both reduce atherogenesis in mice, and protect vascular cells from oxidative stress (Dreger *et al.* 2009; Wilck *et al.* 2012).

UPR activation occurs in atherosclerosis, and in particular can underlie cell death in macrophages, VSMCs and endothelial cells (reviewed in Scull & Tabas, 2011). Defects in proteostasis also have major influences on other signalling pathways that underlie ageing; for example, NADP oxidases are integral signalling elements of the UPR during ER stress, with Nox4 and Nox2 regulating apoptosis after ER stress (reviewed in Laurindo *et al.* 2014). In addition, H_2O_2 is generated by the UPR in response to specific stressors, indicating that the ER surface provides a platform to spatially organize specific oxidative signalling events (Wu *et al.* 2010).

Deregulated nutrient sensing. Dietary restriction results in activation of a number of protective pathways, including those mediated through insulin-like growth factor-1 (IGF1)/Akt, 5'-AMP-activated protein kinase (AMPK) and the sirtuin family of histone deacetylases. The effectors of these pathways include forkhead box protein O (FOXO) proteins, mammalian target of rapamycin (mTOR) and the master regulator of metabolism, PGC1α (Fig. 2). In contrast, atherosclerosis is associated with extensive deposition and storage of both intracellular and extracellular lipid, resulting in signalling a state of nutrient 'excess'.

Although there are multiple and conflicting signals, atherosclerosis is associated with down-regulation of the IGF1 receptor, and subsequent reduction in Akt signalling and consequent activation of FOXO proteins (Patel *et al.* 2001; Allard *et al.* 2008). Loss of Akt1 results in increased atherosclerosis with multiples features of vulnerable plaques (Fernandez-Hernando *et al.* 2007, 2009), a situation that is reversed by overexpression of Akt1 (Tucka *et al.* 2014). Atherosclerosis is also associated with down-regulation of sirtuin 1 (Gorenne *et al.* 2013), which results in increased atherosclerosis, but also a more unstable plaque phenotype and aneurysm formation. In contrast, overexpression of sirtuin 1 results in reduced atherosclerosis, in part via effects on plasma lipids (Miranda *et al.* 2015). Oxidised LDL inhibits mTOR, which increases autophagy, whilst mTOR activation inhibits plaque EC death and atherosclerosis in ApoE$^{-/-}$ mice (Peng *et al.* 2014). Finally, knockout of PGC1α is associated with increased oxidative stress, mitochondrial abnormalities, reduced telomerase activity, reduced sirtuin 1 and the antioxidant catalase, and results in increased senescence-associated-beta-galactosidase (SAβG) staining in mouse arteries and VSMC senescence in culture (Xiong *et al.* 2013).

Mitochondrial dysfunction. DNA damage occurs in both genomic and mitochondrial DNA (MtDNA); the latter is particularly susceptible to damage partly owing to the

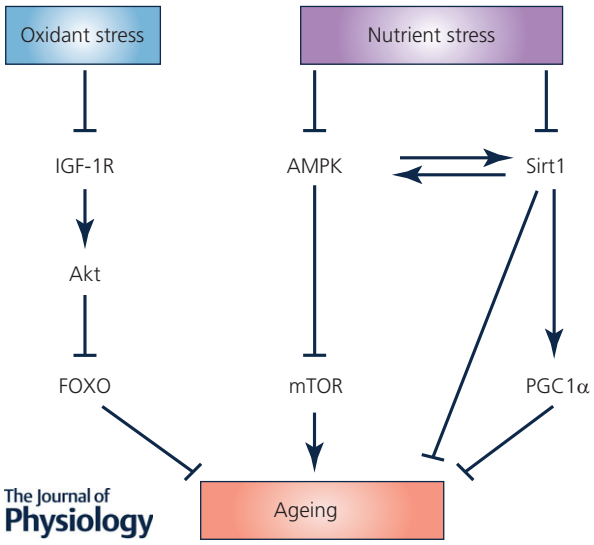

Figure 2. Signalling pathways associated with nutrient excess
A full explanation is provided in the text.

absence of protective histones within the DNA duplex, but also as a result of the close proximity to the ROS-producing inner mitochondrial membrane. MtDNA damage occurs in both cells in atherosclerotic plaque and circulating cells in patients with severe coronary artery disease (CAD) (Botto *et al.* 2005; Yu *et al.* 2013). Similarly, MtDNA damage is an early event in atherosclerosis in experimental animals, and has been detected in arteries, circulating cells and other organs during atherogenesis (Ballinger *et al.* 2002; Yu *et al.* 2013).

The 'free-radical theory of ageing' has been one of the major concepts behind both chronological and biological ageing, where ageing is due to an accumulation of oxidative DNA damage, predominantly driven by ROS. Mitochondrial DNA damage and dysfunction can increase ROS generation, and one of the difficulties has been separating the effects of mitochondrial ROS from loss of other mitochondrial functions. Increased vessel wall ROS and/or reduced ROS scavenger function promote atherosclerosis, and affect multiple aspects of VSMC biology, including cell proliferation, migration and release of pro-inflammatory cytokines, controlled by a number of redox-regulated pathways that show abnormal activity in ageing (see Li, 2010 for review). However, recent studies show that MtDNA damage is sufficient to cause mitochondrial dysfunction during atherogenesis without increased ROS, suggesting that MtDNA damage may have a causal role in atherosclerosis. For example, $PolG^{-/-}$ mice deficient in the mitochondrial polymerase-γ proof-reading enzyme (which accumulate extensive mtDNA damage and defects in oxidative phosphorylation) exhibited increased atherosclerosis associated with increased apoptosis and VSMC senescence with no change in ROS. $PolG^{-/-}$ bone marrow-derived cells altered plaque characteristics, with increased necrotic core and reduction in the relative fibrous cap area, features associated with plaque vulnerability (Yu *et al.* 2013). $PolG^{-/-}$ monocytes also showed increased expression of a number of pro-inflammatory cytokines, suggesting that mitochondrial DNA damage may promote atherosclerosis in part through inflammation (Yu *et al.* 2013).

Cellular senescence. DNA damage checkpoints delay the cell cycle, providing sufficient time for cells to repair lesions. However, ineffective DNA damage repair can lead to irreversible growth arrest, termed cellular senescence. In addition, the normal inability of eukaryotic cells to maintain the telomere length results in progressive telomere shortening with each division. The telomere is sensed as a strand break at a critical telomere length or structure, resulting in DDR activation and replicative senescence. Growth inhibition usually occurs in G_1, and is regulated by pathways mediated by $p16^{INK4a}$/retinoblastoma protein (pRB) and p53/p21 (Campisi, 2005). In addition, a range of extrinsic and intrinsic stimuli, particularly those causing intracellular oxidative stress, can induce DNA damage and senescence acutely (Herbert *et al.* 2008), termed 'stress-induced premature senescence' (SIPS) (Toussaint *et al.* 2000). Both SIPS and replicative senescence induce the same phenotype of cells, and are mediated by some of the same pathways, although telomere length may be unaffected in SIPS. Aged vessels and atherosclerotic lesions show SAβG-positive VSMCs, ECs and monocyte/macrophages, which are rarely seen in their respective young and normal counterparts (Minamino *et al.* 2002; Matthews *et al.* 2006), reinforcing the idea that atherosclerosis is associated with premature cellular senescence.

Similarly, human VSMCs derived from both aged vessels and advanced atherosclerotic plaques show markers of senescence in culture, including reduced proliferation, prolonged population doubling times (O'Brien *et al.* 1993; Bennett *et al.* 1995), and decreased S-phase and increased G_1 percentages consistent with a G_1 growth arrest (Bennett *et al.* 1995). Growth arrest is associated with increased expression of the cyclin-dependent kinase inhibitors $p16^{INK4a}$ and p21 (Matthews *et al.* 2006), decreased cyclin D and cyclin E (O'Sullivan *et al.* 2003), and pRB hypophosphorylation (Bennett *et al.* 1998; O'Sullivan *et al.* 2003; Matthews *et al.* 2006), similar to that seen in normal human VSMCs undergoing replicative senescence. Importantly, these cell cycle regulators become potential markers of vascular cell senescence.

Stem cell exhaustion. A number of studies suggest that some cells expressing VSMC or EC markers in the atherosclerotic plaque and normal vessel may be derived from bone marrow-derived cells (BMCs) or endothelial progenitor cells (EPCs) that migrate and integrate into the vessel wall. Although the proportion of ECs and VSMCs from these sources appears small (Bentzon *et al.* 2006; Hagensen *et al.* 2010, 2012), dysfunctional EPCs (Heiss *et al.* 2005) and impaired BMC migration and adhesion (Heiss *et al.* 2005; Xu *et al.* 2011) are seen in both aged and atherosclerotic mouse models. ECs from aged animals show reduced expression of cell surface markers and cytokines for chemotaxis such as CXCR4 (Shao *et al.* 2011; Xu *et al.* 2011) and decreased HIF-1α (Bosch-Marce *et al.* 2007; Chang *et al.* 2007), as well as increased oxidative stress and inflammation (Zhang *et al.* 2009; Carracedo *et al.* 2011).

There is similar controversy over the presence of stem or progenitor smooth muscle cells in the vessel wall. VSMCs are not post-mitotic, and can differentiate and adopt the phenotype of many different mesenchymal cells, including bone, cartilage and adipose tissue. Furthermore, populations of cells in the adventitia that demonstrate stem cell markers have been shown to contribute to neointima formation after vein grafting in mice, although

their contribution to primary atherosclerosis appears low (Hu *et al.* 2004; Tsai *et al.* 2012). More recently, a population of multipotent vascular stem cells has been suggested that can also give rise to more specialised tissue including neurons and Schwann cells in addition to VSMCs (Tang *et al.* 2012). Studies such as these suggest that there is a vascular stem cell niche that may become exhausted through multiple divisions and/or damage (reviewed in Psaltis & Simari, 2015).

Altered intercellular communication. Cells undergoing senescence in response to a persistent DDR induce a 'senescence associated secretory phenotype' (SASP), which involves secretion of pro-inflammatory cytokines, chemokines, growth factors and proteases (Passos *et al.* 2010; van Deursen, 2014). Evolutionarily the SASP may have a beneficial effect on the local environment, such that increased cell proliferation, migration or differentiation might promote tissue repair. However, immune cells can be mobilised by the SASP, which might promote atherosclerosis (Fyhrquist *et al.* 2013). In addition, senescent cells reinforce their phenotype to non-senescent neighbouring cells via autocrine and paracrine effects (Acosta *et al.* 2008, 2013; van Deursen, 2014), which may promote atherosclerosis. For example, the matrix metalloproteinases including MMP-1, MMP-3 and MMP-10 are SASP cytokines (Davalos *et al.* 2010) which contribute to extracellular matrix degradation and weakening of the vessel wall. Although, recent studies show that a persistent DDR stimulates interleukin-6 and -8 secretion, the mechanism through which DNA damage induces the SASP is unclear (Rodier *et al.* 2009).

Functional consequences of cell ageing

Ageing-associated changes are observed in multiple cell types and are conserved across species, from rodents to primates. As well as loss of normal cellular function, these changes result in specific consequences in each cell type, which may be directly pro-atherogenic.

Endothelial cells. Aged ECs become flatter and enlarged and have an increasingly polypoid nucleus, all features associated with cellular senescence. These changes are accompanied by modulation in cytoskeleton integrity, proliferation, angiogenesis and cell migration. For example, senescent ECs show attenuated endothelial NO production (Sato *et al.* 1993) and increased endothelin-1 release (Donato *et al.* 2009); late passage ECs also show increased expression of the adhesion molecules VCAM-1 and ICAM-1, increased activation of NF-κB, and increased susceptibility to apoptosis (Khaidakov *et al.* 2011). Thus, EC senescence is associated with loss of EC function and a shift towards a pro-inflammatory and pro-apoptotic state, predicted to enhance monocyte migration into the vessel wall.

Vascular smooth muscle cells. VSMCs in human plaques or derived from plaques show reduced proliferation, early senescence and increased susceptibility to apoptosis (Bennett *et al.* 1995). These properties would reduce the ability to repair plaques that undergo rupture. Aged rodent aortas also show increased levels of interleukin-6 (IL-6) and aged aortic VSMCs have a higher basal secretion of IL-6 than young VSMCs as part of the 'senescence-associated secretory phenotype' (SASP). Moreover, aged VSMCs exhibit upregulation of chemokines (CCL2), adhesion molecules (e.g. ICAM-1), and innate immune receptors (e.g. Toll-like receptor 4) (Song *et al.* 2012). These properties generate a pro-inflammatory environment, further promoting migration of inflammatory cells. VSMC senescence thus promotes atherosclerosis progression and inhibits plaque repair.

Inflammatory cells. As mentioned above, leukocyte telomere length is inversely associated with atherosclerosis in population studies, suggesting that leukocyte ageing has direct functional consequences that might promote atherosclerosis. Monocytes from patients with atherosclerosis demonstrate an increased burst of free radicals on activation and increased secretion of a number of cytokines, including MCP-1, IL-6, IL-1β and TNF-α (Calvert *et al.* 2011). Importantly, many of these differences are also observed in aged *versus* young monocytes and can be recapitulated by agents that disrupt telomeres (Calvert *et al.* 2011). Thus, there is direct evidence that ageing promotes pro-inflammatory changes in monocytes/macrophages that are relevant to atherosclerosis (Calvert *et al.* 2011). Coupled with altered adhesion molecules on aged ECs, ageing would be predicted to promote both migration and activation of macrophages within plaques.

Conclusions

There is extensive evidence of accelerated biological ageing in atherosclerosis, with the large majority of the 'hallmarks of ageing' being present in advanced plaques. However, cellular ageing is not just a marker of disease, but contributes directly to both atherogenesis and the clinical sequelae of atherosclerosis, including plaque rupture that leads to myocardial infarction and stroke. The causal role of cellular ageing in atherosclerosis indicates that treatments aimed at both prevention of cellular ageing and reducing its consequences are viable therapeutic targets. Indeed, some of the current standard therapies for atherosclerosis may work in part by ameliorating the local arterial ageing process. For example, statins

upregulate TRF2 (Spyridopoulos *et al.* 2004), decrease DNA damage by accelerating DNA damage repair (Pernice *et al.* 2006; Mahmoudi *et al.* 2008), and suppress oxidative stress in part by increasing antioxidant defences. Statins can also delay VSMC replicative senescence and reduce DNA damage *in vivo* in atherosclerosis (Mahmoudi *et al.* 2008). Similarly, angiotensin converting enzyme inhibitors reduce oxidative stress and subsequent DNA damage. The next step is to determine whether compounds directly targeted at ageing can safely slow the development of atherosclerosis.

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Additional information

Competing interests

None.

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