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Abstract

In the present review, we describe the causes and consequences of loss of vascular smooth muscle cells (VSMCs) or their function in advanced atherosclerotic plaques, and discuss possible mechanisms such as cell death or senescence, and induction of autophagy to promote cell survival. We also highlight the potential use of pharmacological modulators of these processes to limit plaque progression and/or improve plaque stability. VSMCs play a pivotal role in atherogenesis. Loss of VSMCs via initiation of cell death leads to fibrous cap thinning and promotes necrotic core formation and calcification. VSMC apoptosis is induced by pro-inflammatory cytokines, oxidized LDL, high levels of nitric oxide and mechanical injury. Apoptotic VSMCs are characterized by a thickened basal lamina surrounding the cytoplasmic remnants of the VSMC. Inefficient clearance of apoptotic VSMCs results in secondary necrosis and subsequent inflammation. A critical determinant in the VSMC stress response and phenotypic switching is autophagy, which is activated by various stimuli, including reactive oxygen and lipid species, cytokines, growth factors and metabolic stress. Successful autophagy stimulates VSMC survival, whereas reduced autophagy promotes age-related changes in the vasculature. Recently, an interesting link between autophagy and VSMC senescence has been uncovered. Defective VSMC autophagy accelerates not only the development of stress-induced premature senescence but also atherogenesis, albeit without worsening plaque stability. VSMC senescence in atherosclerosis is likely a result of replicative senescence and/or stress-induced premature senescence in response to DNA damaging and/or oxidative stress-inducing stimuli. The finding that VSMC senescence can promote atherosclerosis further illustrates that normal, adequate VSMC function is crucial in protecting the vessel wall against atherosclerosis.

1. Introduction

Despite the significant therapeutic advances in cardiology, atherosclerosis remains a leading cause of acute cardiovascular death. Vascular smooth muscle cells (VSMCs) play a pivotal role in atherogenesis.[1] The majority of VSMCs in the plaque derive from the medial layer of the blood vessel. However, VSMCs in the media are surrounded by a basement membrane, which provides a brake on VSMC proliferation and migration. Extracellular proteolysis is therefore necessary before VSMCs can migrate into the intima. Loss of the surrounding basement membrane induces a shift from a contractile to a synthetic phenotype, which is associated with decreased expression of VSMC differentiation marker proteins, an increase in proliferation rate and an increase in synthesis of extracellular matrix. In animal models, VSMCs migrate to the intima in response to platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGFβ). VSMCs are beneficial and essential for plaque stability, since they are the main source of collagen production in the fibrous cap and responsible for its tensile strength. Accordingly, loss of VSMCs via initiation of cell death has detrimental effects, leading to fibrous cap thinning, necrotic core formation and calcification.[1] In the present review, we describe the causes and consequences of loss of VSMCs or their function in advanced plaques, and discuss possible mechanisms including cell death and senescence, in addition to the protective effects of autophagy induction. Moreover, we highlight the potential use of pharmacological modulators of the abovementioned processes to prevent plaque rupture and its clinical complications.

2. VSMC death in atherosclerosis

Cell death in atherosclerosis was first reported by the vascular pathologist Virchow in 1858. He stated that atherosclerotic plaques are formed by a dynamic interplay between cell replication and cell death. At that time, plaque cell death was generally recognized as non-programmed necrosis. However, apart from necrosis, apoptosis is a major event in the pathophysiology of atherosclerosis and most evidence for the activation of this cell death pathway in advanced plaques was gathered in the early 1990s. Both apoptosis and necrosis have been identified in different cell types of the plaque including macrophages, endothelial cells and VSMCs. However, the consequences of cell death in atherosclerosis depends on the specific cell type involved, the developmental stage of the plaque, and of course, the type of cell death itself.

2.1. VSMC Apoptosis in atherosclerosis

Apoptosis is the best-studied form of programmed cell death (*Figure 1*) and can be morphologically distinguished by cellular shrinkage, chromatin condensation, membrane blebbing and internucleosomal DNA fragmentation. Several studies have shown evidence for apoptotic cell death in both human and animal atherosclerotic plaques.[2, 3] Apoptotic VSMCs can be distinguished from other cell types by the

presence of a thickened basal lamina surrounding the cytoplasmic remnants of the VSMC, which are called matrix vesicles (*Figure 2*). The level of apoptosis is low in early plaques but increases as lesions become more advanced. Thus, VSMC death differentiates the initial fatty streaks from fibro-atheroma. Fatty streaks have a cellular-rich background mainly constituted of VSMCs. In contrast, the lipid core of fibro-atheroma is mainly acellular due to the progressive in situ disappearance of VSMCs whereas migrated VSMCs proliferate and recover the lipid core forming a fibro-cellular cap. Usually the « necrotic » core in more complex plaques is an haemorrhagic core.[4] Overall, the apoptotic index of advanced plaques averages at 1-2%.[2] Apoptotic cells mostly accumulate in the fibrous cap, at sites of rupture, close to lipid deposits and necrotic cores, and in the presence of inflammatory cells.

2.1.1. Causes of VSMC apoptosis in atherosclerosis

VSMC apoptosis is induced by pro-inflammatory cytokines, oxidised low density lipoprotein (oxLDL), high levels of nitric oxide (NO) and mechanical injury. Pro-inflammatory cytokines secreted by plaque macrophages (e.g. tumour necrosis factor (TNFα) and T cells (e.g. interferon (IFN)-γ) sensitize VSMCs to apoptosis, for example that mediated by the death receptor Fas (CD95) by promoting Fas trafficking to the cell surface.[5] The Fas receptor /Fas Ligand pathway is also involved in oxLDL-induced apoptosis in VSMCs,[6] and plaque VSMCs are very sensitive to apoptosis mediated through the tumour suppressor gene p53. High levels of p53 induce apoptosis in human plaque VSMCs in low serum conditions but not in normal medial VSMCs, but p53 activation sensitizes human VSMCs to Fas-mediated apoptosis by transiently increasing surface Fas expression by transport from the Golgi apparatus.[7] Moreover, sensitivity to p53-mediated apoptosis increases when VSMCs are driven to proliferate.[8] Paradoxically, high levels of apoptosis together with low levels of proliferation have been observed in VSMCs of human plaques.[9] This may be explained by the predominance of the hypophosphorylated form of the tumour suppressor retinoblastoma protein in plaque VSMCs that promotes cellular senescence (vide infra).[10] By profiling apoptosis-related genes in human atherosclerotic plaques, death-associated protein (DAP) kinase was identified as a highly expressed protein in foam cells of VSMC origin, even though the exact role of DAP kinases in atherosclerosis still needs to be unravelled.[11] Finally, VSMCs in human fatty streaks highly express the pro-apoptotic gene Bax, which renders them more sensitive to undergo apoptosis via the mitochondrial pathway.[2]

2.1.2. Consequences of VSMC apoptosis in atherosclerosis

Apoptosis in VSMCs of advanced plaques contributes to increased plaque vulnerability, stenosis and medial degeneration.[12, 13] Moreover, VSMC apoptosis promotes plaque thrombogenicity by exposing phosphatidylserine on the surface of apoptotic cells that can act as a substrate for thrombin generation and activation of the coagulation cascade.[14] Remnants of apoptotic VSMCs remain in the plaque as matrix vesicles and can act as nucleating structures for bone formation, resulting in plaque micro-calcifications.[15] These spotty or micro-calcifications are associated with enhanced plaque progression

and have the potential to increase the occurrence of plaque rupture via the induction of biomechanical stress on the fibrous cap.[16] Furthermore, human VSMCs are potent phagocytes of apoptotic VSMCs, though their phagocytic capacity is reduced by hyperlipidaemia.[17] Inefficient clearance of apoptotic VSMCs results in secondary necrosis and subsequent interleukin 1-driven inflammation.[17] Thus, VSMC apoptosis also contributes to plaque inflammation.

2.1.3. Pharmacological modulation of apoptosis in VSMCs

Since caspases play an essential role in apoptosis, inhibition of these proteases has been investigated as an approach to reduce apoptotic cell death and stabilize atherosclerotic plaques. Local treatment with the broad caspase inhibitor zVAD-fmk reduces VSMC apoptosis and neointimal hyperplasia.[18] Nevertheless, caspase-3 deficient VSMCs show a higher susceptibility to necrosis. In line with these findings, caspase-3 deletion promotes primary necrosis and results in increased plaque size in mice.[19] Thus, inhibition of apoptosis seems unfavourable to reduce atherosclerosis.

2.2. VSMC necrosis in atherosclerosis

Necrosis is characterised by a gain in cell volume (oncosis) and swelling of organelles, followed by rupture of the plasma membrane and release of the intracellular contents, which evokes an inflammatory response. Necrotic cells are less efficiently cleared by phagocytes compared to apoptotic cells. Though necrosis was originally considered as an accidental, non-programmed form of cell death (due to physio-chemical stress), accumulating evidence indicates that necrosis can be regulated via different signalling transduction pathways leading to the formation of a necrosome (comprising receptor interacting protein kinase-1 [RIPK1], RIPK3 and its substrate mixed lineage kinase like [MLKL]) that induces RIPK1 kinase activity and RIPK3 kinase activity-dependent necroptosis, opening of the mitochondrial permeability transition pore or poly(ADP)ribose polymerase 1 (PARP1) overactivation (parthanatos).[20] Ferroptosis is a form of regulated necrotic cell death characterised by accumulation of lipid peroxidation products and lethal amounts of reactive oxygen species (ROS) derived from iron metabolism.[21]

2.2.1. Causes of VSMC necrosis in atherosclerosis

Necrosis can be stimulated by oxLDL, depending on its dose and degree of oxidation.[22] For example, highly oxidised LDL induces necrosis rather than apoptosis, while mildly oxidised LDL induces ER stress/apoptosis rather than necrosis through calcium intracellular deregulation and the intrinsic mitochondrial pathway in VSMCs.[23, 24] High levels of ROS in the plaque cause irreversible oxidative damage to key cellular components such as DNA, lipids and proteins. Severe DNA damage can evoke PARP overactivation[25] whereas the generated lipid peroxidation products can disrupt organelles and the plasma membrane, leading to necrosis. Moreover, depletion of the cytosolic ATP pool switches cell fate from apoptosis to necrosis. In particular, loss of function of ATP-dependent ion channels in the plasma

membrane causes cell swelling due to the uncontrolled influx of cations, and finally rupture of the plasma membrane, while increased intracellular calcium levels induce necrosis via activation of calcium-dependent proteases (e.g. calpains) and/or increased mitochondrial ROS generation (in case of mitochondrial Ca^{2+}-overload).[26] Finally, defective efferocytosis of apoptotic cells in advanced atherosclerotic plaques leads to accumulation of these cells, which will eventually undergo secondary necrosis.[27]

2.2.2. Consequences of VSMC necrosis in atherosclerosis

When plaque cells die by necrosis, they release their lipid and inflammatory content and form the necrotic core. Though it is presumed that the necrotic core contains mainly macrophage debris,[28] also plaque VSMCs have been shown to exhibit morphological features typical of necrosis. The size of the necrotic core has been associated with the incidence of plaque rupture.[29] Advanced-stage human plaques exhibit necrotic cores larger than 10% of the total lesion area. In 66% of the lesions that show plaque rupture, the necrotic core size is increased and occupies more than 25% of the total lesion. Thus, the larger the necrotic core, the higher the risk of plaque rupture. The release of pro-inflammatory molecules by necrotic cells illustrates that plaque necrosis can directly promote plaque inflammation. For example, the release of the nuclear protein HMGB1 (high mobility group box 1) stimulates macrophage inflammation[30] by acting as a DAMP (damage-associated molecular pattern). Via binding with the receptor for advanced glycation end products (RAGE) or members of the toll-like receptor (TLR) family (e.g. TLR4), HMGB1 triggers pro-inflammatory cytokine production in macrophages. Necrotic VSMCs have been shown to secrete IL1α whereas during secondary necrosis, both IL1α and IL1β are released.[17] Both cytokines act on the surrounding viable VSMCs, stimulating them to produce pro-inflammatory cytokines such as IL6 and monocyte chemoattractant protein 1 (MCP1), amplifying inflammation.[17] Necrotic cells also release matrix metalloproteinases, which are able to degrade the ECM and contribute to plaque instability. Finally, the necrotic core contains high concentrations of tissue factor, released from foam cells undergoing secondary necrosis, which facilitates thrombus formation after plaque rupture.[22]

2.2.3. Pharmacological modulation of necrosis in VSMCs

The understanding that necrosis can occur in a highly regulated manner has led to the development of small molecule inhibitors of regulated necrosis. Although the effects on VSMC necrosis were not specifically investigated, they are worth mentioning. NecroX-7 is able to improve plaque stability in Apolipoprotein E knockout ($ApoE^{-/-}$) mice by reducing the necrotic core area, increasing the collagen content and generating a less inflammatory plaque phenotype.[31] This molecule exerts these beneficial effects by reducing mitochondrial ROS production, which leads to an inhibition of oxidative stress-induced necrosis.[31] Another interesting compound is necrostatin-1 (Nec-1), which inhibits necroptosis

by targeting RIPK1.[22] Atherosclerosis-prone mice treated with Nec-1 showed a significant reduction in plaque burden and more stable lesions characterized by a smaller necrotic core and higher VSMC content.[32] Overall, these data suggest that targeting necrosis might be a valuable approach to stabilize atherosclerotic plaques.

3. VSMC autophagy in atherosclerosis

Autophagy is a "housekeeping" subcellular process for lysosome-mediated turnover of damaged cytosolic material, and is tightly regulated by more than 30 highly conserved AuTophaGy related (ATG) genes. Autophagic degradation is important in maintaining normal cellular homeostasis and energy balance, and basal autophagy is necessary for mediating appropriate vascular function. Autophagy exists in three major forms: microautophagy, chaperone-mediated autophagy, and macroautophagy. The most prevalent and best-studied form is macroautophagy, hereafter referred to as autophagy. In the general form of the process, cytoplasmic cargo targeted for destruction is sequestered inside double-membrane vesicles called autophagosomes and delivered to the lysosome by fusion for breakdown. The degradation products are transported back to cytoplasm where they can be reused for biosynthesis or energy production. Autophagy modulation has a major effect on vessel wall function and the initiation or the progression of vascular diseases including atherosclerosis.[33]

3.1. Evidence of VSMC autophagy in atherosclerosis

Previous work using transmission electron microscopy showed characteristics of autophagy in dying VSMCs of both human and cholesterol-fed rabbit atherosclerotic plaques, in particular vesicular structures containing amorphous material.[34, 35] Additional ultrastructural data of the autophagic process in human atherosclerotic plaques established that VSMCs, macrophages and ECs may display autophagic activation.[36] However, further studies are needed to determine whether these observations were at an early or a more complicated stage of the lesion. Interestingly, autophagy is a critical determinant in VSMC phenotypic switching and the cellular stress response. Following vascular injury, VSMCs modify their phenotype from the baseline contractile state to a proliferative synthetic state, a mechanism stimulated by growth factors such as PDGF. Treatment of VSMCs with PDGF induces autophagy through a 5' adenosine monophosphate-activated protein kinase (AMPK)-independent and mammalian target of rapamycin (mTOR)-independent mechanism, resulting in the removal of contractile proteins and the upregulation of osteopontin (OPN) and vimentin.[37] Similarly, secreted Sonic hedgehog (Shh) increases the expression of the synthetic phenotype markers and promotes autophagy in an Akt-dependent manner.[38] Conversely, the pharmacological inhibition of autophagy by 3-methyladenine (3-MA) or spautin-1 stabilizes the contractile phenotype and prevents PDGF or Shh-induced VSMC proliferation.[37] Vascular calcification is driven by VSMCs and leads to the induction of active osteogenic differentiation of VSMCs within the vessel wall.[39] Recently, autophagy has been

identified as a novel adaptive mechanism that protects against phosphate-induced VSMC calcification by regulating apoptosis and the release of mineralizing matrix vesicles from VSMCs.[40] Drugs activating autophagy such as atorvastatin and telmisartan protect VSMCs from transforming growth factor beta 1 (TGF-β1)-stimulated calcification[41] and lipid accumulation-induced foam cell formation.[42] Moreover, autophagy regulates VSMC contraction and relaxation. Defective autophagy in VSMCs affects the contractile capacity and Ca^{2+} mobilization. Loss of this process leads to changes in Ca^{2+} homeostasis and enhanced vascular reactivity, independent of alterations in the contractile apparatus.[43] Taken together, these findings demonstrate that autophagy is indispensable during the process of VSMC plasticity and phenotypic changes.

3.2. Causes of VSMC autophagy in atherosclerosis

Autophagy is activated in VSMCs by various stimuli and stressors including reactive oxygen and lipid species, cytokines, growth factors and metabolic stress (*Table 1*).

3.2.1. Reactive oxygen and lipid species

Autophagy could be activated by ROS, oxLDL and secondary products of the oxidative degradation of lipids.[42] For example, the stimulation of VSMCs with atherogenic oxidized lipids results in an increased expression of autophagy-related proteins and in the formation of autophagosomes, but the outcomes depend on the concentration and the oxidation level of LDL. Modest concentrations (10-40 $\mu g.mL^{-1}$) of highly oxidized LDL augment autophagy and apoptosis in VSMCs, whereas higher concentrations ($\geq$ 60 $\mu g.mL^{-1}$) trigger high levels of apoptosis but disrupt autophagy, showing that the stress response induced by autophagy becomes dysfunctional when a certain threshold of cell injury is achieved.[44] Conversely, the selective removal of damaged mitochondria through autophagy in human VSMCs exposed to high concentrations of mildly oxidized LDL acts as a safeguard mechanism against apoptosis.[45]

In lipoproteins, the oxidative degradation of lipids produces bioactive lipid intermediates and peroxidation end products. These reactive lipid species, including free aldehydes (e.g. 4-hydroxynonenal, acrolein) and to a minor extent lipid hydroperoxides, induce a strong activation of autophagy.[46] Similarly, the stimulation of human VSMCs with 7-ketocholesterol (7-KC), one of the major oxysterols present in atherosclerotic plaques, generates signs of ubiquitination and characteristics of autophagy.[35] The molecular mechanism by which 7-KC induces VSMC autophagy involves the production of ROS mediated by Nox4, which triggers the activity of ATG4B.[47] Prevention of autophagy aggravates both endoplasmic reticulum (ER) stress and cell death in response to 7-KC, while up-regulation of autophagic activity by rapamycin has opposite effects.[47]

3.2.2. Cytokines and growth factors

Depending on the settings, inflammatory cytokines (e.g. INFγ, TNFα) and CD40-CD40L interactions are able to trigger autophagy or conversely repress it.[48] TNFα, a factor secreted by VSMCs and inflammatory cells, increases the formation of autophagosomal vesicles and the expression of the autophagy markers LC3-II and beclin-1 in VSMCs isolated from human atheromatous lesions.[49] Furthermore, treatment of cultured VSMCs with the cytokine OPN increases the expression of autophagy-related genes and the formation of autophagosomes, resulting in VSMC death.[50] Since autophagy triggered by OPN is impaired by inhibition of the integrin and CD44 families of cell surface receptors and p38 MAPK signalling, it is conceivable that OPN stimulates autophagy via these pathways. Additionally, treatment of VSMCs with angiotensin II induces autophagy in a dose- and time-dependent manner. Upon vascular injury or chronic arterial disease, VSMCs are exposed to increased concentrations of growth factors. Besides PDGF or Shh that trigger an autophagy process involved in the proliferation of VSMCs,[37, 38] insulin-like growth factor-1 (IGF-1) promotes cell survival through the inhibition of autophagy in plaque VSMCs.[49]

3.2.3. Metabolic stress

Although nutrient deprivation is a powerful activator of autophagy, nutrient excess induces autophagy in vascular tissues of mice fed a high-fat diet[51], which is associated with insulin resistance and ER stress. The administration of osteocalcin, a secretory product from osteoblasts which is an important regulator of glucose and lipid metabolism, not only attenuates autophagy and ER stress but also rescues the impaired insulin signalling and restores defective insulin sensitivity in VSMCs.[51]

The formation of AGEs, which is driven by hyperglycaemia and oxidative stress, plays a crucial role in the onset and the progression of oxidative-based vascular disease. Autophagy is triggered by AGEs and contributes to cell proliferation through ERK, JNK and p38 signalling in rat aortic VSMCs, suggesting that AGEs-induced autophagy accelerates the development of atherosclerosis in diabetic patients.[52]

Hypoxia present in advanced atherosclerotic plaques triggers IP neovascularization. In human pulmonary hypoxic VSMCs, the activation of autophagy prevents the proliferation of VSMCs. Hypoxia activates autophagy through the phosphorylation of the metabolic sensor AMPK, whereas the suppression of AMPK alpha 1 inhibits hypoxia-induced autophagy and cell death.[53]

3.3. Consequences of VSMC autophagy

Currently, many studies are performed to understand the functional role of autophagy in vascular disease. Given that dysregulated autophagy has been described in several cardiovascular diseases, it is still debated whether autophagy is a protective or harmful mechanism in vascular pathology (*Table 1*). However, the general consensus is that successful autophagy promotes VSMC survival. It has been shown *in vitro* that VSMC death induced by low concentrations of statins or by free cholesterol excess

could be reduced by oxysterol or rapamycin pre-treatment which both induced autophagy.[35, 54] Moreover, autophagy may be crucial for VSMC survival under conditions associated with extreme lipid peroxidation. It has been shown that aldehyde-modified proteins can be removed by autophagy, whereas these compounds trigger apoptosis in VSMCs if autophagy is impaired.[46] Loss- or gain-of-expression approaches have demonstrated that selective uptake of mitochondria by autophagy, also called mitophagy has critical consequences on VSMC fate by enhancing apoptosis or by favouring cell survival. For example, PINK1 or PARK2 knockdown by small-interfering RNAs increases the cytotoxic response of human VSMCs challenged with oxLDL, whereas PINK1 or PARK2 overexpression has cytoprotective effects.[45] Even though autophagy is generally believed as a protective process against atherogenic stressors, massive stress and excessively activated autophagy are able to induce autophagic death of VSMCs,[55] resulting in decreased synthesis of collagen leading to plaque destabilisation.

With increasing age, vascular autophagy flux seems to decline and may contribute to age-related endothelial dysfunction and arterial stiffness.[56] Indeed, the addition of two autophagy inducers trehalose or spermidine reverses age-associated increases in aortic pulse wave velocity and normalises levels of aortic collagen I and AGEs.[57] Therefore, reduced autophagy may be an important mechanism underlying age-related changes in the vasculature. Recently, an interesting link between VSMC senescence and autophagy has been uncovered. Deletion of the essential autophagy gene Atg7 in murine VSMCs causes accumulation of SQSTM1/p62 and accelerates the development of stress-induced premature senescence as shown by cellular and nuclear hypertrophy, CDKN2A-RB-mediated G1 proliferative arrest and senescence-associated β-galactosidase activity.[58] Moreover, VSMC-specific deletion of Atg7 in $ApoE^{-/-}$ mice accelerates atherosclerotic plaque development after 10 weeks of high-fat diet. The atherosclerotic lesions in VSMC-specific Atg7 knockout mice exhibit increased plaque cell death, plaque macrophages, fibrous cap thickness, and COL3A1 collagen content. After 14 weeks of high-fat diet, lesions show no significant differences in plaque size, macrophage content, cell necrosis or apoptosis in both groups of mice, indicating that defective autophagy in VSMCs accelerates atherogenesis without worsening plaque stability.

3.4. Pharmacological modulation of autophagy in VSMCs

Currently, the perspective of modulating autophagy through pharmacological approaches represents an attractive strategy to treat or prevent cardiovascular disease. In cultured VSMCs, autophagy stimulated by valproic acid diminishes calcification by reducing the release of matrix vesicles.[40] On the contrary, the use of three pharmacologically unrelated inhibitors of autophagy including 3-MA, spautin-1 and bafilomycin A1 stabilizes the contractile VSMC phenotype. The significant effectiveness of spautin-1 *in vitro* holds that this agent might be suitable to avoid the phenotype switch and the proliferation observed in vascular restenosis *in vivo*. In addition, rapamycin and rapalogs (e.g. everolimus) that induce

autophagy through mTOR inhibition, have been tested as atherosclerotic plaque stabilizing drugs. In atherosclerotic plaques from cholesterol-fed rabbits, stent-based delivery of everolimus leads to a prominent reduction in the macrophage content without changing the amount of VSMCs.[59] This effect could be due to the high metabolic activity of macrophages that renders them more susceptible to inhibitors of protein synthesis than VSMCs. In addition, the inhibition of protein translation can make VSMCs less responsive to death due to the transition from a contractile to a quiescent phenotype. Recently, Luo et al reported that moderate autophagy induction by low-dose rapamycin improves plaque stability in fat-fed ApoE$^{-/-}$ mice.[60] Rapamycin treatment reduced plaque necrotic core size and overall plaque burden in the thoracic aorta, while lesional SMC and collagen content were increased. Interestingly, treatment with higher doses of rapamycin did not exert any additional beneficial effects. Conversely, ApoE$^{-/-}$ mice treated with 3-MA exhibited an opposite plaque phenotype.[60] Spermidine, an endogenous biological polyamine that exhibits broad longevity-extending activities via the induction of autophagy, reduces lipid accumulation and necrotic core formation in atherosclerotic plaques of fat-fed ApoE$^{-/-}$ mice.[61] Additionally, spermidine attenuates lipid accumulation by stimulating cholesterol efflux via autophagy activation.[61] Hence, a moderate induction of autophagy in VSMCs could protect against cell death and vascular calcification, which are all events that promote atherosclerotic plaque stabilisation (*Figure 3*). In contrast, defective autophagy induces VSMC senescence and changes in VSMC phenotype or contractile function, thus promoting neointima formation, plaque progression and instability (*Figure 3*). Overall, in view of the fundamental importance of autophagy in many cellular functions, the pharmacological modulation of autophagy undoubtedly represents a promising tool for the treatment of vascular diseases.

4. VSMC senescence in atherosclerosis

Cellular senescence is defined as an irreversible loss of proliferation potential. After a series of replications, cells cease to divide as a result of telomere shortening/uncapping and undergo so-called 'replicative senescence'. Growth arrest is initiated upon activation of a DNA damage response (DDR) pathway that engages the tumour suppressor gene p53 and the cyclin-dependent kinase inhibitor (cdki) p21, and is subsequently stabilized by activation of the cdki p16 and hypophosphorylation of the Retinoblastoma protein (pRB), resulting in silencing of proliferation-promoting genes.[62] Cellular senescence can also be induced by various stimuli such as oncogene activation (oncogene-induced senescence) or DNA damaging agents and oxidative stress. This latter type of senescence, called 'stress-induced premature senescence (SIPS)', can involve both p53/p21 and p16/RB pathways.[63] In addition to the permanent growth arrest, senescent cells acquire a wide range of morphological and functional changes, including adopting a flattened and hypertrophic morphology and expression of 'senescence-associated β-galactosidase' (SAβG), a pH-sensitive enzyme activity that reflects an increase in lysosomal mass. One of the main features of senescent cells is the development of a 'senescence-

associated secretory phenotype' (SASP), which results in secretion of a range of pro-inflammatory cytokines (e.g. IL6, IL8), chemokines (e.g. MCP1) and proteases (e.g. MMPs), and which contributes to the effect of cellular senescence on tissue inflammation.[64] Cellular senescence has been recognised as an important hallmark of aging and is considered to exert functional consequences on VSMCs comprising the atherosclerotic plaque.[65]

4.1. Evidence of VSMC senescence in atherosclerosis

The occurrence of VSMC senescence in human atherosclerosis has become widely accepted, although the lack of a common universal marker for cell senescence in tissues challenges its identification and quantification. To date, we do not know the exact number of senescent VSMCs present in the atherosclerotic lesion, and whether it correlates with the stage of plaque development.

4.1.1. Human VSMC senescence

The observation of a low level of proliferation in VSMCs derived from atherosclerotic plaques compared with cells from the normal arterial media was one of the first indications that plaque VSMCs undergo premature senescence.[10] Human plaque VSMCs are characterized by higher expression levels of p16 and p21, hypophosphorylation of pRB, a large flattened cell shape and SAβG activity, as compared to normal VSMCs.[66] Immunohistochemical analysis of advanced human lesions shows co-localisation of p16 and p21 with SAβG-positive VSMCs, while these markers were not observed in normal healthy vessels.[67] Moreover, there is direct evidence for replicative VSMC senescence in human atherosclerosis.[67] For example, telomeres in intimal VSMCs are significantly shorter compared with medial VSMCs and barely evident in advanced plaque VSMCs, indicating that telomere length is inversely correlated with the severity of atherosclerosis.[67] The attrition of telomeres may be due to the increased rates of VSMC proliferation during early lesion development, although telomeres undergo accelerated shortening after oxidative stress.[67]

4.1.2. Mouse VSMC senescence

The number of studies using atherosclerosis-prone mice to examine senescence, and VSMC senescence in particular, are extremely scarce. The lack of extensive evidence for VSMC senescence in murine atherosclerosis partially originates from the general concept that mice are incapable of undergoing replicative senescence due to their very long telomeres (40-60 kb versus 5-15 kb in humans).[68] However, this does not exclude the possibility that murine cells can undergo other forms of cellular senescence. To date, two independent studies report evidence for DNA damage-induced senescence in VSMCs in atherosclerotic mice involving telomeric dysfunction.[69, 70] However, the incidence of other forms of SIPS has been insufficiently demonstrated, and SAβG-activity is often used as a (exclusive) marker of senescence. For instance, 18 month-old ApoE$^{-/-}$ mice fed a cholesterol-rich diet display high levels of

aortic SAβG activity, which was not observed in age-matched controls.[71] High SAβG activity is also present in LDLR knockout mouse plaques after short periods of fat feeding, suggesting that senescence may occur much earlier than originally thought.[72] However, a word of caution is required when interpreting SAβG staining in atherosclerotic tissue. Plaque macrophages and foam cells may show false-positive SAβG staining due to their high lysosomal content.[73] Moreover, cell-specific lineage markers are required to identify the cell type that expresses SAβG activity. Different studies report SAβG activity in intimal VSMCs ranging from a few %[58] to more than 20%,[72] and telomere-associated DNA damage foci (TAF) have been observed in 45% of all medial VSMCs in fat-fed ApoE$^{-/-}$ mice.[70] We therefore recommend using a combination of different cell cycle markers such as p16 and p21 together with SAβG and other markers to identify senescent cells in plaques.

Taken together, there is evidence for VSMC senescence in murine atherosclerosis involving DNA damage, telomeric dysfunction, and activation of different SIPS pathways, but senescence in atherosclerotic mice requires further in-depth validation, especially when testing potential anti-senescent therapies.

4.2. Causes of VSMC senescence in atherosclerosis

Based on the current knowledge, VSMC senescence in (human) atherosclerosis is likely a result of replicative senescence (with telomere shortening) and/or SIPS in response to DNA damaging and/or oxidative stress-inducing stimuli known to be abundantly present in atherosclerotic lesions.

4.2.1. Defective telomere integrity

Reduction in telomere length, changes in the telomere structure (e.g. telomeric fusion), or loss of telomere binding factors (e.g. shelterin complex members including TRF1, TRF2) are all potential triggers of senescence.[74] In contrast, telomeres are maintained by the telomerase enzyme that adds new (5'-TTAGGG-3') repeat sequences onto the 3' end of the telomeres. Both plaque and normal aortic VSMCs have low telomerase activity.[67] However, elevating telomerase expression in VSMCs restores proliferation, rescues senescence and prolongs lifespan, despite short telomeres, suggesting that both telomere length and telomerase activity are important determinants of VSMC senescence in human atherosclerosis. Additionally, it has been suggested that failure to maintain telomeric integrity is critical to induction of senescence rather than loss of telomere length.[75] For example, loss of function of the shelterin protein TRF2 in murine VSMCs induces senescence and DNA damage, whereas overexpression of TRF2 has opposite effects.[69] Moreover, analysis of human atherosclerotic plaques reveals reduced expression of TRF2 in plaque VSMCs, which is associated with increased DNA damage, implying a role for TRF2 in plaque VSMC senescence.

4.2.2. Oxidative stress

Oxidative stress has been suggested to play a role in cellular senescence and in human aging in general.[76] Aged murine VSMCs exhibit a lower proliferation potential and higher levels of ROS compared to younger VSMCs, and are associated with increased oxidant-induced damage.[77] ROS generate a spectrum of DNA damage including DNA strand breaks and DNA base modifications that trigger a DNA damage response pathway, with activation of the effector proteins p53/p21 via the ATM kinase signalling cascades. Although exposure of human VSMCs to pro-oxidants generally triggers SIPS,[78] chronic oxidative stress accelerates replicative senescence as evidenced by telomere shortening and reduced telomerase activity.[67] Conversely, suppression of oxidative stress, either by culturing vascular cells in hypoxic conditions[79] or treatment with antioxidants[80], delays replicative senescence and extents lifespan. Since the majority of ROS originate from mitochondria as reactive by-products of the electron transport chain, it is assumed that loss of mitochondrial function accelerates senescence due to increased ROS generation and the concomitant accumulation of oxidative mtDNA damage.[81] In addition to the 'free radical theory of aging', direct damage to the electron transport chain (ETC) has been shown to induce senescence.[82] Although the specific signalling pathway is still unclear, increased ROS production as a result of a damaged ETC is speculated to be a plausible cause of cellular senescence.[82] Hence, given that mitochondrial dysfunction has been associated with the progression of atherosclerosis, loss of mitochondrial function may promote atherogenesis, at least in part, by promoting VSMC aging.

4.3. Consequences of VSMC senescence in atherosclerosis

Increasing evidence suggests that VSMC senescence contributes to the progression and destabilization of atherosclerotic plaques (*Figure 4*). Due to the loss of proliferative potential of senescent cells, senescence can trigger plaque instability directly by reducing the VSMC content of the fibrous cap and compromising its repair after rupture. However, more recent evidence suggests a more active role of VSMC senescence in promoting plaque destabilisation by driving plaque inflammation and matrix degradation.

4.3.1. Inflammation

Senescent human VSMCs secrete a wide range of SASP mediators (e.g. IL6, IL8, MCP1) in a IL1α-dependent manner, while the expression of anti-inflammatory molecules (e.g. RANTES and IL1R2) is reduced.[83] Secretion of SASP factors from senescent VSMCs promotes chemotaxis of monocytes/macrophages, and stimulates adjacent non-senescent VSMCs or ECs to release cytokines and express adhesion molecules.[83] Furthermore, senescent VSMCs upregulate multiple inflammasome components that can further compromise plaque stability.[83] Analysis of human carotid plaques shows increased expression of IL1α and IL6 in SAβG-positive VSMCs within the fibrous cap, and reveals the presence of CD68-positive cells in their vicinity, which might reflect the increased chemotaxis of

macrophages in response to the SASP.[83] These data strengthen our knowledge on how VSMC senescence may actively contribute to plaque instability by promoting inflammation. In this way, even a low number of senescent VSMCs may have a major impact on disease progression.

The mechanism of the SASP in VSMCs is incompletely understood, but activation of the oncogene ras induces senescence in human VSMCs, which is associated with an ERK-dependent increase in pro-inflammatory cytokine expression.[84] Introduction of ras into rat carotid injured arteries accelerates intimal VSMC senescence, which is accompanied by increased accumulation of macrophages.[84] Analysis of human atherosclerotic plaques also reveals co-localisation of intimal SAβG-positive VSMCs with activated ERK and IL1β, suggesting that VSMC senescence plays a role in vascular inflammation through ERK and interleukin release.[84]

4.3.2. Plaque vulnerability

The discovery of the SASP as a key feature of human senescent VSMCs led to the concept that senescent VSMCs can promote plaque vulnerability in part through the secretion of matrix-degrading proteases.[83] Senescent human VSMCs produce less collagen compared to normal VSMCs[83], which can further jeopardize plaque stability. VSMCs also adopt an 'osteoblast-like' phenotype and enhance their susceptibility to calcification by upregulating the expression of different osteoblastic genes when undergoing replicative senescence.[85] Although it is still not completely clear whether vascular calcification contributes to plaque vulnerability, it is closely associated with a high incidence of cardiovascular events,[86] and so these findings further support the involvement of senescent VSMCs in the progression of atherosclerosis.

4.3.3. Plaque development

Although senescent VSMCs are more frequent in advanced lesions, VSMC senescence may also regulate atherosclerotic plaque development, at least in mice. For example, VSMC-specific overexpression of TRF2 in ApoE$^{-/-}$ mice prevents senescence, reduces DNA damage and improves several features of plaque vulnerability.[69] In contrast, VSMC-specific TRF2 mutant mice show increased atherosclerosis, which is associated with increased necrotic core formation, DNA damage and senescence; analysis of serum levels of typical SASP proteins and macrophage lesional content suggested that the observed increase in atherosclerosis was not due to a systemic SASP. Therefore, VSMC senescence can promote atherosclerosis in mice possibly independently of a SASP, and illustrates again that normal, adequate VSMC function is absolutely crucial in protecting the vessel wall against atherosclerosis.

4.4. Pharmacological modulation of VSMC senescence

Many biochemical agents that harbour anti-senescence potential are effective in the treatment of (experimental) atherosclerosis.[87] For example, a newly developed set of drugs called "senolytics" that selectively target and eliminate senescent cells by inducing apoptosis may have therapeutic potential in atherosclerosis. This strategy is based on the observation that senescent cells often develop apoptotic resistance by the upregulation of different senescence cell anti-apoptotic pathways (SCAPs).[88] The first drugs to target SCAPs were the tyrosine kinase inhibitor dasatinib and the flavonoid quercetin. Dasatinib and quercetin in combination reduce the number of TAF-positive senescent VSMCs in the media of fat-fed ApoE$^{-/-}$ mice, and diminish aortic calcification and osteogenic signalling in the intima, although they do not attenuate atherosclerotic plaque size.[70] However, it is not clear whether senolytics truly result in senescent cell killing. Moreover, both compounds are nonspecific.[89] Other senolytic drugs inhibit the anti-apoptotic members of the Bcl2-family (e.g. Navitoclax). Their major side effect is thrombocytopenia, which severely limits their suitability in patients with cardiovascular disease, although off-target effects of senolytics may be restricted by the use of cell-permeable peptides that do not block the entire function of the protein, but only interfere with certain protein-protein interactions to push the cells into a pro-apoptotic route.[89]

Removal of senescent cells can also be achieved by boosting the immune system. Senescent cells may express specific surface markers[90] that can be recognised by different cells of the immune system and subsequently killed.[91] In this way, antibodies raised against these antigens could drive cytotoxic T cells or NK cells to clear senescent cells, or to deliver senolytic drugs via nanoparticles.[92] These findings raise the question whether the accumulation of senescent cells within the vasculature is partially due to their defective clearance by professional phagocytes, as described for apoptotic bodies in advanced plaques.

An alternative approach to target senescent cells is by blocking the SASP. Targeting every SASP factor seems unachievable and likely unnecessary, and inhibition of the upstream signalling pathways (such as NFkβ and MAPK) can provoke off-target effects since they are not exclusively linked to senescence. In contrast, drugs such as rapamycin can dampen the SASP of senescent fibroblasts, presumably by inhibition of mTOR-dependent IL1α mRNA translation and the JAK/STAT3 pathway.[93] Besides its anti-inflammatory property, rapamycin exerts anti-senescence effects in VSMCs via inhibition of the mTOR pathway[94, 95] - which is known to be overactivated in senescent cells - and activation of autophagy.[60, 95] Although senolytic therapies are promising to treat age-related diseases such as atherosclerosis, a lot of questions still remain to be answered. For example, prevention may be easier than treatment, so when should senolytic therapy start (early vs. advanced plaques), and what would be the dosage regimen (chronic vs. intermittent)? Mouse studies suggest that removal of senescent cells may both delay atherogenesis and the progression of established plaques,[72] but whether they are powerful enough to reverse established atherosclerotic plaques in humans remains to be determined.

5. Crosstalk between VSMC autophagy, senescence and death: fight, adapt or die?

When VSMCs in the arterial wall or atherosclerotic lesions are challenged by stressful insults (DNA damage, ROS, oxidized lipids, inflammatory cytokines, …), the VSMC has mainly 3 options: either fight (autophagy), adapt (senescence) or die (apoptosis/necrosis) (*Figure 5*). The choice for each of these strategies likely depends on the strength of the stimulus, the time of exposure, the presence of co-stimuli, the 'health state' of the VSMC (e.g. VSMC foam cell) and its phenotype (contractile vs. synthetic). By activating the autophagic pathway, VSMCs tend to overcome toxic insults by facilitating their removal to promote cell survival. During senescence, VSMCs adapt to the stressful condition by undergoing a proliferative arrest. Although they remain alive, they endure drastic metabolic and morphological changes. When the toxic insults are too powerful or persistent, the cell chooses to die. Whether the cell dies by apoptosis or necrosis, both pathways are tightly controlled and should not be considered as an accidental form of cell demise but as a well-considered strategy. All three pathways have been shown to be intimately connected which greatly affects VSMC behaviour and vitality. For example, moderate activation of autophagy safeguards VSMCs from cell death and has been shown to suppress VSMC senescence[60, 94, 95]. Senescent cells are often characterised by apoptotic resistance due to the upregulation of anti-apoptotic proteins. During apoptotic cell death, autophagic pathways are blocked, e.g. due to the inhibition of Beclin-1 by Bcl2[96], to ensure complete execution of death. The connection between the 3 pathways becomes even more apparent when 1 of them fails. For instance, blockage of autophagy by genetic disruption of essential autophagy proteins promotes senescence in VSMCs.[58, 95] Conversely, stimulation of autophagy by rapamycin attenuates VSMC senescence.[60, 95]

Based on the known interplay between these 3 pathways and the data gathered from the analysis of human and mouse atherosclerotic samples, it is tempting to speculate that the decline in VSMC autophagy in advanced atherosclerotic plaques is associated with the increased incidence of cell death and senescence. Moreover, the imbalance between these 3 pathways likely favours the conversion of a stable atherosclerotic plaque into an unstable phenotype. Although senescent VSMCs seem harmless at first sight - due to their inability to proliferate and their resistance to apoptosis - they create an inflammatory environment via their SASP. Hence, from a translational perspective, small molecules or drugs that stimulate (moderate) autophagy but inhibit senescence at the same time, such as rapamycin, should be considered as having promising therapeutic value for the future treatment of atherosclerosis.

6. Conclusion

In conclusion, VSMC apoptosis is induced in atherosclerotic plaques by several stimuli including oxLDL. Inefficient clearance of apoptotic VSMCs results in secondary necrosis and inflammation. However, exposure of VSMCs to modest amounts of oxLDL enhances autophagy, whereas high

concentrations impair autophagy. Accordingly, successful autophagy promotes VSMC survival, whereas reduced autophagy underlies age-related changes in the vasculature. Indeed, defective VSMC autophagy accelerates the development of stress-induced premature senescence, which promotes atherosclerosis. Therefore, adequate VSMC function is crucial in protecting the vessel wall against atherosclerosis.

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Table 1 : Autophagic stimuli and consequences on VSMC fate.

Autophagic stimuli	Effect on autophagy	VSMC fate	References
Growth factors and cytokines			
• PDGF • Shh	Activation	Cell survival	37, 38
• Osteopontin • Angiotensin II • TNFα	Activation	Cell death	49, 50
• IGF-1/insulin	Inhibition	Cell survival	49
Reactive oxygen and lipid species			
• ROS • 7-ketocholesterol • oxidized LDL • Lipid peroxides (4-HNE, POVC, acrolein)	Activation	Cell survival	35, 42, 44-47
Metabolic stress			
• Hypoxia • Nutrient excess • AGEs	Activation	Cell survival	51-53

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Figure legends

Figure 1. Induction, mechanism and consequences of VSMC apoptosis in atherosclerosis. The death receptor pathway is activated by binding of ligands (TRAIL, TNFα, FasL) to their receptors (DR4/5, TNFR1, Fas). These receptors recruit adapter proteins and activate caspase-8 which in turn activates the effector caspases (caspase-3,6,7). In the mitochondrial pathway, death signals reach the mitochondria where pro-apoptotic proteins (Bax/Bak) give rise to mitochondrial outer membrane permeabilisation (MOMP) and subsequent release of cytochrome c, which forms a complex with Apaf-1 and pro-caspase-9 (apoptosome), leading to caspase-9 activation and consecutive activation of the effector caspases. Cytochrome c release is prevented by the anti-apoptotic proteins Bcl-2 and Bcl-XL. The mitochondrial pathway may also be triggered by p53 upon DNA damage. p53 is able to increase Bax/Bak oligomerisation and inhibits the anti-apoptotic effects of Bcl-2 and Bcl-XL. Furthermore, p53 can initiate Fas-mediated apoptosis by increasing Fas expression at the cell surface. Crosstalk between the apoptosis pathways involves caspase-8-mediated cleavage of Bid into tBid, which may promote cytochrome c release. VSMCs in an atherosclerotic plaque are subjected to several apoptotic stimuli. TNFα initiates apoptosis via binding with TNFR1. Both TNFα and IFNγ promote Fas trafficking to the cell surface, thereby activating the death receptor pathway. oxLDL enhances VSMC apoptosis by induction of Fas, FasL and p53. Plaque VSMCs show high Bax expression and have an increased sensitivity for p53-mediated apoptosis. Apoptosis of plaque VSMCs leads to unstable plaques characterized by a thin fibrous cap, micro-calcifications, a large necrotic core and a high degree of inflammation.

Figure 2. Identification of VSMC apoptosis in advanced human atherosclerotic plaques. A. Terminal deoxynucleotidyl transferase end labelling (TUNEL, red) combined with a staining for collagen IV (blue) shows the presence of an apoptotic VSMC (arrow head) in the fibrous cap (FC) which could be identified by a TUNEL-positive nucleus surrounded by a cage of basal lamina. NC: necrotic core, scale bar: 30µm. **B.** Transmission electron microscopy of an advanced plaque clearly demonstrates the disintegration of an apoptotic VSMC in matrix vesicles (arrow heads), encaged by a thick basal lamina (BL). Scale bar: 2µm

Figure 3. Effect of functional and dysfunctional VSMC autophagy on atherosclerotic plaque stability. By modulating VSMC phenotype and viability autophagy may be a critical regulator of atherosclerotic plaque stability. Effective autophagy in VSMC has been shown to promote the conversion of contractile VSMC to a synthetic phenotype and to inhibit cell death. Conversely, impaired autophagy appears to induce VSMC senescence and cell death which contributes to plaque instability.

Figure 4. Causes and consequences of VSMC senescence in atherosclerosis. VSMCs in atherosclerotic plaques undergo senescence in response to different triggers such as DNA damage, oxidative stress and loss of telomere integrity. Senescent VSMCs are characterised by upregulation of the cell cycle inhibitors p16 and/or p21, cellular and nuclear hypertrophy, and accumulation of senescence-associated β-galactosidase (SAβG). Human VSMCs undergoing replicative senescence can adopt an 'osteoblast-like' phenotype and promote vascular calcification. Human senescent VSMCs also produce less collagen but secrete more extracellular matrix degrading proteases (e.g. MMPs). Combined with their inability to proliferate, VSMC senescence promotes fibrous cap thinning by compromising its repair after rupture. Secondly, senescent VSMCs secrete many pro-inflammatory cytokines as part of their senescence-associated secretory phenotype (SASP). These cytokines stimulate adjacent non-senescent endothelial cells (ECs) and VSMCs to express adhesion molecules and release cytokines, and/or trigger monocyte (MΦ) recruitment. Through the establishment of the SASP, senescent VSMCs contribute to plaque inflammation and further jeopardize plaque stability.

Figure 5: Crosstalk between autophagy, senescence and cell death. VSMCs in the arterial wall or atherosclerotic lesions are continuously challenged by stressful insults such as DNA damaging molecules, ROS, oxidized lipids, inflammatory cytokines, hypoxia, etc. and will respond in 3 different ways: either fight (autophagy), adapt (senescence) or die (apoptosis/necrosis). By activating autophagy, VSMCs try to overcome toxic insults by facilitating their removal to promote cell survival. During senescence, VSMCs adapt to the stressful conditions by undergoing a proliferative arrest. Although they remain alive, they experience pronounced metabolic and morphological changes. When the toxic insults are too powerful or persistent, the cell chooses to die either by apoptosis or necrosis. All the three pathways are interconnected and negatively control each other. Moderate activation of autophagy safeguards VSMCs from cell death while during apoptosis, autophagic pathways are blocked to ensure complete execution of death. Stimulation of autophagy supresses VSMC senescence whereas inhibition of autophagy promotes it. Senescent cells are often characterised by apoptotic resistance due to the upregulations of anti-apoptotic proteins.