6

Endothelial and Vascular Smooth Cell Dysfunctions: A Comprehensive Appraisal

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1. Introduction

Cardiovascular disease (CvDs) such as coronary artery disease, hypertension, congestive heart failure and stroke are the leading causes of death and disability in the Western World (Madamanchi et al., 2005; Thom, 1989). The majority of CvDs results from complication of atherosclerosis. Prevention of cardiovascular events is therefore urgently needed and is one of the major recent challenges of medicine. New molecular imaging approaches featuring the assessment of inflammatory processes in the vascular wall (on top of existing anatomic and functional vessel imaging procedures) could emerge as decisive tools for the understanding and prevention of cardiovascular events (Schafers et al., 2010).

2. Atherosclerosis

Atherosclerosis is a progressive disease, affecting medium and large-sized arteries, characterized by patchy intramural thickening of the subintimal that encroaches on the arterial lumen (Bonomini et al., 2008). The atherosclerosis plaque is characterized by an accumulation of lipid in the artery wall, together with infiltration of macrophages, T cells and mast cells, and the formation by vascular smooth muscle cells (VSMCs) of a fibrous cap composed mostly of collagen. Early lesions called "fatty streaks" consist of sub-endothelial deposition of lipid, macrophage foam cells loaded with cholesterol and T cells. Over time, a more complex lesion develops, with apoptotic as well as necrotic cells, cell debris and cholesterol crystals forming a necrotic core in the lesion. This structure is covered by a fibrous cap of variable thickness, and its "shoulder" regions are infiltrated by activated T cells, macrophages and mast cells, which produce proinflammatory mediators and enzymes (Hansson et al., 2006). Plaque growth can cause stenosis (narrowing of the lumen) that can contribute to ischemia in the surrounding tissue (Hansson & Hermansson, 2011).
Although the pathophysiological mechanisms underlying atherosclerosis are not completely understood, it is widely recognized that both inflammation and oxidative stress play important roles in all of the phases of atherosclerosis evolution (Cipollone et al., 2007).

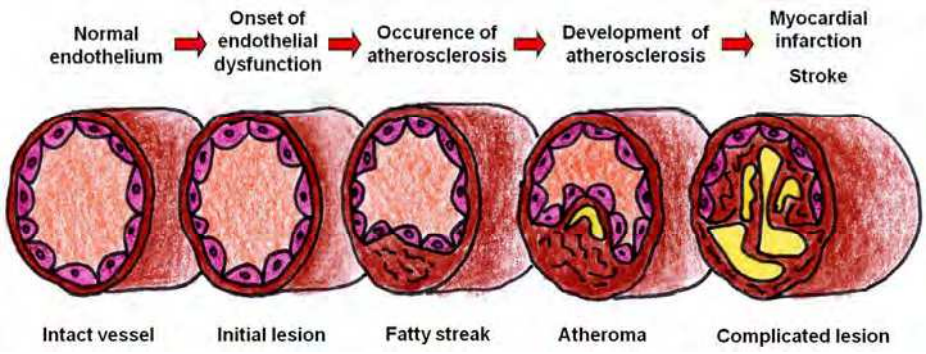

Fig. 1. Steps involved in atherosclerosis progression from endothelial dysfunction to cardiovascular complication.

2.1 Atherosclerosis and oxidative stress

Oxidative stress can be defined as an "imbalance between oxidants and antioxidants in favor of the oxidants, potentially leading to damage" (Sies, 1991). Age, gender, obesity, cigarette smoking, hypertension, diabetes mellitus and dyslipidemia are known atherogenic risk factors that promote the impairment of endothelial function, smooth muscle function and vessel wall metabolism. These risk factors are associated with an increased production of reactive oxygen species (ROS) (Antoniades et al., 2003). ROS play a physiological role in the vessel wall and participate as second messengers in endothelium-dependent function, in smooth muscle cells and endothelial cells (ECs) growth and survival, and in remodelling of the vessel wall. Each of these responses, when uncontrolled, contributes to vascular diseases (Fortuño et al., 2005; Griendling & Harrison, 1999; Irani, 2000; Taniyama & Griendling, 2003).

In the vasculature wall, ROS are produced by all the layers, including tunica intima, media and adventitia. ROS include superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH), nitric oxide (NO), and peroxynitrite ($ONOO^-$) (Lakshmi et al., 2009). The major vascular ROS is O_2^-, which inactivates NO, the main vascular relaxing factor, thus impairing relaxation (Cai & Harrison, 2000; Kojda & Harrison, 1999). Dismutation of O_2^- by superoxide dismutase (SOD) produces H_2O_2, a more stable ROS, which, in turn, is converted to water by catalase and glutathione peroxidase. H_2O_2 and other peroxides appear to be important in the regulation of growth-related signalling in VSMCs and inflammatory responses in vascular lesions (Irani, 2000; Li, P.F. et al., 1997). High levels of O_2^-, the consequent accumulation of H_2O_2 and diminished NO bioavailability play a critical role in the modulation of vascular remodelling. Finally, $ONOO^-$, resulting from the reaction between O_2^- and NO, constitutes a strong oxidant molecule, which is able to oxidize proteins, lipids and nucleic acids and then causes cell damage (Beckman & Koppenol, 1996; Fortuño et al., 2005).

There are several potential sources of ROS production. In cardiovascular disease the sources include xanthine oxidase, cyclooxygenase, lipooxygenase, mitochondrial respiration, cytochrome P450, uncoupled nitric oxide synthase (NOS) and NAD(P)H oxidase. They have been identified as sources of ROS generation in all type of vasculature. These sources may contribute to ROS formation, depending on cell type, cellular activation site and disease

context. Numerous studies have shown that various physiological stimuli that contribute to pathogenesis of vascular disease can induce the formation of ROS (Lakshmi et al., 2009). ROS have detrimental effects on vascular function through several mechanisms. First, ROS, especially hydroxyl radicals, directly injure cell membranes and nuclei. Second, by interacting with endogenous vasoactive mediators formed in ECs, ROS modulate vasomotion and the atherogenic process. Third, ROS peroxidize lipid components, leading to the formation of oxidized lipoproteins (LDL), one of the key mediators of atherosclerosis (Bonomini et al., 2008).

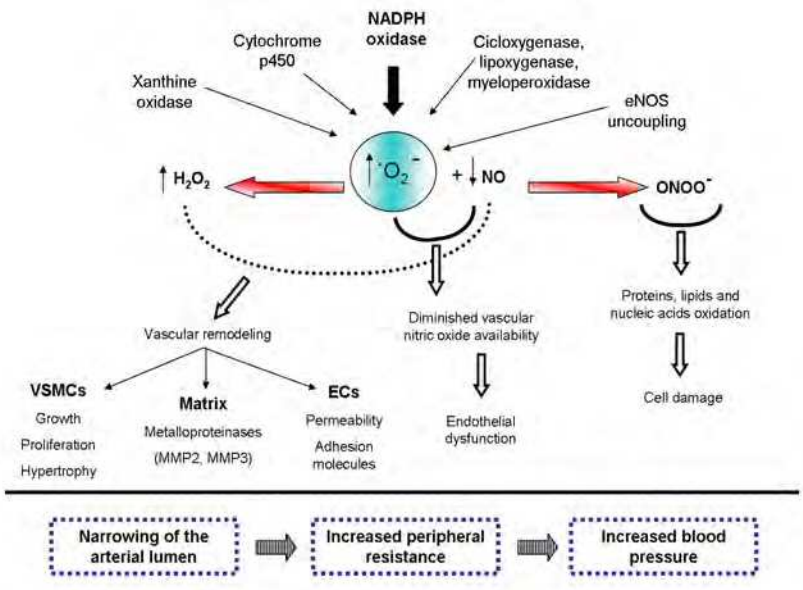

Fig. 2. Potential sources of ROS production in atherosclerosis progression.

Cholesterol is transported in the blood by LDL. These particles contain esterified cholesterol and triglycerides surrounded by a shell of phospholipids, free cholesterol and apolipoprotein B100 (ApoB100). Circulating LDL particles can accumulate in the intimal, the innermost layer of the artery. Here ApoB100 binds to proteoglycans of the extracellular matrix (ECM) through ionic interactions (Tabas et al., 2007). This is an important initiating factor in early atherogenesis (Skålen et al., 2002; Steinberg, 2009; Witztum & Steinberg, 2001). As a consequence of this subendothelial retention, LDL particles are trapped in the tunica intima, where they are prone to oxidative modifications caused by enzymatic attack of myeloperoxidase (Heinecke, 2007) and lipoxygenases, or by ROS such as hypoclorous acid (HOCl), phenoxyl radical intermediates or $ONOO^-$ generated in the intimal during inflammation and atherosclerosis (Hansson & Hermansson, 2011).

Oxidized LDL (Ox-LDL) has several biological effects (Madamanchi et al., 2005); it is pro-inflammatory; it causes inhibition of endothelial NOS (eNOS); it promotes vasoconstriction and adhesion; it stimulates cytokines such as interleukins (ILs) and increases platelet aggregation. Ox-LDL-derived products are cytotoxic and induce apoptosis. Ox-LDL can adversely affect coagulation by stimulating tissue factor and plasminogen activator

inhibitor-1 (PAI-1) synthesis. Another atherogenic property of Ox-LDL is its immunogenicity and its ability to promote retention of macrophages in the arterial wall by inhibiting macrophage motility (Singh & Jialal, 2006). In addiction, Ox-LDL stimulates VSMCs proliferation (Stocker & Keaney, 2004). Thus, intimal thickening further reduces the lumen of blood vessels, leading to further potentation of hypertension and atherosclerosis (Singh & Jialal, 2006). With ongoing oxidation, the physicochemical properties gradually change, including alterations in charge, particle size, lipid content and other features. The precise nature of each of these alterations obviously depends on the oxidizing agent. For all these reasons, Ox-LDL is not a defined molecular species but is instead a spectrum of LDL particles that have undergone a variety of physicochemical changes (Hansson & Hermansson, 2011).

2.2 Atherosclerosis and inflammation

Inflammation participates in atherosclerosis from its inception onwards. Fatty streaks do not cause symptoms, and may either progress to more complex lesions or involute. Fatty streaks have focal increases in the content of lipoproteins within regions of the intimal, where they associate with components of the ECM such as proteoglycans, slowing their egress. This retention sequesters lipoproteins within the intimal, isolating them from plasma antioxidants, thus favoring their oxidative modification (Kruth, 2002; Packard & Libby, 2008; Skålen et al., 2002). Oxidatively modified LDL particles comprise an incompletely defined mixture, because both the lipid and protein moieties can undergo oxidative modification. Constituents of such modified lipoprotein particles can induce a local inflammatory response (Miller et al., 2003; Packard & Libby, 2008).

Vascular ECs function to prevent clotting of blood and adhesion of blood cells to the endothelial cells, in addition to playing the role of a barrier, as a cell monolayer, to prevent blood constituents from invading the vascular wall. When ECs are injured or activated by various coronary risk factors, infections or physical stimuli, adhesion molecules become expressed in ECs, and peripheral monocytes adhere to the endothelial cell surface. Adhesion molecules are broadly divided into three molecular families: integrin family, immunoglobulin family, and selectin family (L-selectin, Eselectin, P-selectin) (Yamada, 2001).

Chemoattractant factors, which include monocyte chemoattractant protein-1 (MCP-1) produced by vascular wall cells in response to modified lipoproteins, direct the migration and diapedesis of adherent monocytes (Boring et al., 1998; Packard & Libby, 2008). Monocytic cells, directly interacting with human ECs, increase several fold monocyte matrix metalloproteinase (MMP) 9 production, allowing for the subsequent infiltration of leukocytes through the endothelial layer and its associated basement membrane (Amorino & Hoover, 1998; Packard & Libby, 2008) Within the intima, monocytes mature into macrophages under the influence of macrophage colony stimulating factor (M-CSF), which is overexpressed in the inflamed intima. M-CSF stimulation also increases macrophage expression of scavenger receptors, members of the pattern-recognition receptor superfamily, which engulf modified lipoproteins through receptor-mediated endocytosis. Accumulation of cholesteryl esters in the cytoplasm converts macrophages into foam cells, i.e., lipid-laden macrophages characteristic of early-stage atherosclerosis. In parallel, macrophages proliferate and amplify the inflammatory response through the secretion of numerous growth factors and cytokines, including tumor necrosis factor α (TNFα) and IL-1β. Recent

evidence supports selective recruitment of a proinflammatory subset of monocytes to nascent atheroma in mice (Packard & Libby, 2008).

A number of proinflammatory cytokines have been shown to participate in atherosclerotic plaque development, growth and rupture (Dabek, 2010; Libby et al., 2002). Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) seems to be a crucial transcription factor in the cross-talk among cytokines, adhesion molecules and growth factors. On one hand, NF-kB is a major transcription factor leading to cytokine synthesis, and on the other hand, the above mentioned factors keep NF-kB persistently activated in acute coronary syndromes (Dabek, 2010). In atherogenesis, NF-kB before regulates the expression of cyclooxygenases, lipooxygenases, cytokines, chemokines (i.e., MCP-1) and adhesion molecules (Dabek, 2010; Kutuk & Basaga, 2003). Later in the progression of the atherosclerotic lesion, NF-kB regulates gene expression of M-CSF, a factor stimulating infiltrating monocyte differentiation and transformation into "foamy cells", and other genes participating in the transformation (Brach et al., 1991; Dabek, 2010). As stated, atherosclerosis is an inflammatory reaction of the arterial wall. The factors IL-1β, TNF-α, IL-6, IL-12 and interferon γ (IFNγ) are involved in this reaction and their expression is coregulated by NF-kB.

Intracellular matrix degradation is an important process in both plaque development and rupture. The vital factors involved include MMPs, particularly those that are able to break down the vascular base membrane. It has been shown that NF-kB is an essential regulator of MMP gene expression, especially MMP-2 and MMP-9, which are critical in plaque rupture (Bond et al., 1998; Dabek, 2010). Thus, NF-kB regulates the expression of a wide spectrum of atherosclerosis mediating factors. On the other hand, most of these factors also up-regulate NF-kB activity. Increased NF-kB activity was found in unstable regions of atherosclerotic plaques (Brand et al., 1997; Dabek, 2010). The significance of NF-kB activity has been confirmed in some clinical studies as well. Li and colleagues reported significantly increased NF-kB activity in white blood cells from unstable angina patients *vs.* stable angina patients and *vs.* control patients (the lowest activity in the latter) (Li, J.J. et al., 2004).

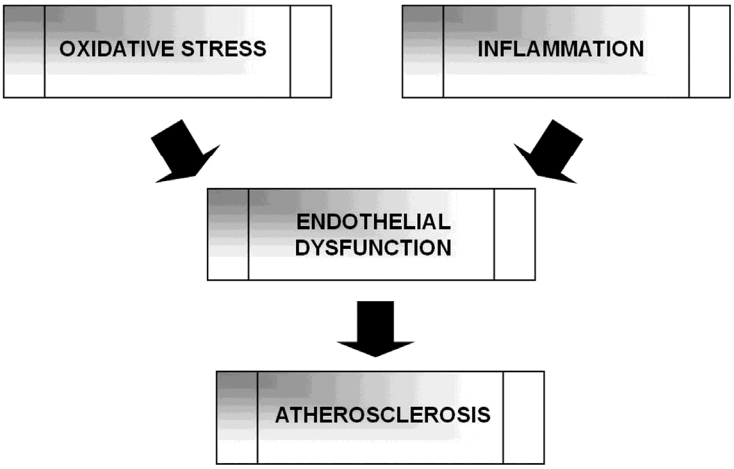

Fig. 3. Role of oxidative stress and inflammation in the early atherosclerosis.

3. Endothelial cells dysfunction in atherosclerosis

The endothelium is responsible for the regulation of vascular tone, the exchange of plasma and cell biomolecules, inflammation, lipid metabolism and modulation of fibrinolysis and coagulation (Andrews et al., 2010). Aging affects many pathways involved in cardiovascular functions and particularly of ECs (Barton, 2010; Virdis et al., 2010). In fact, endothelial-aging is associated with anatomical disruption, morphological abnormalities in ECs size and shape (Haudenschild et al., 1981), susceptibility to apoptosis and abnormal release of EC-derived factors (Barton, 2010). These factors, which are synthetized not only by ECs, but also by VSMCs, are now known to contribute to pathogenetic mechanisms of CVDs (Higashi et al., 2009).

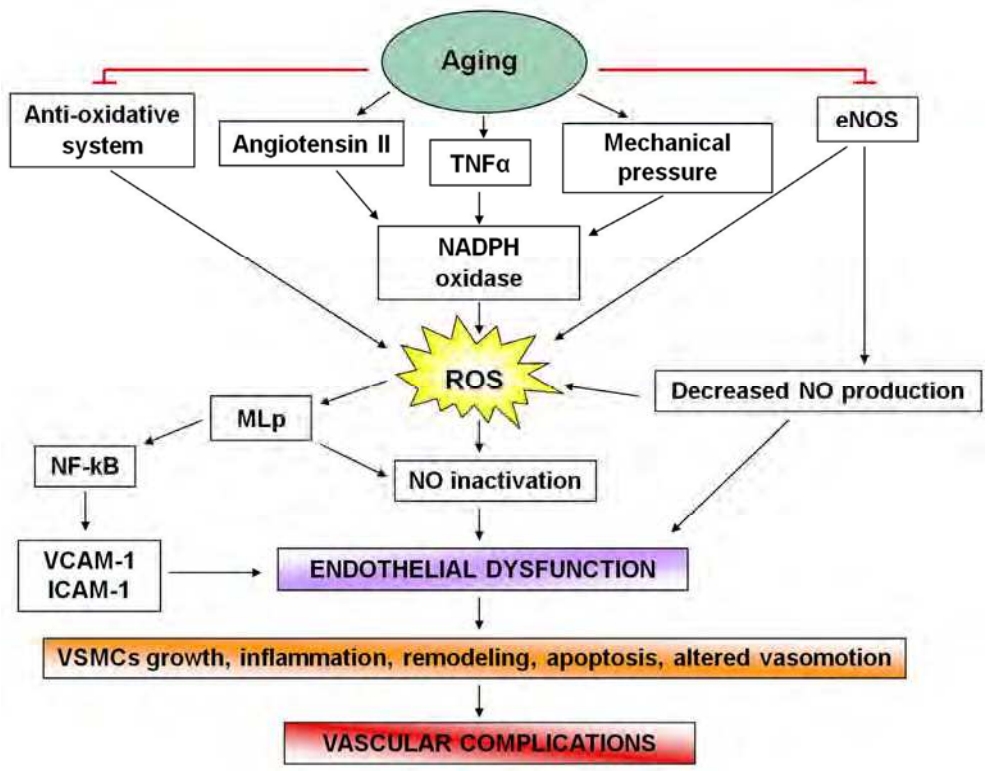

Fig. 4. Central role of ROS in inducing endothelial dysfunction in vascular diseases.

ECs dysfunction, inflammation, oxidative stress and dyslipidaemia are known to play prominent and vital roles not only in the development of atherosclerotic lesions, but also in their progression. (Andrews et al., 2010; Bai et al., 2010; Higashi et al., 2009 ; Virdis et al., 2010). Number of factors and modalities are available to interfere with age related changes in EC function (Barton, 2010; Jensen-Urstad et al., 1999). When endothelial damage compromises the normal vascular function, the intracellular dynamic balance probably leans on an athero-prone phenotype.

Growing evidence indicates that chronic and acute overproduction of ROS activates ECs as pivotal early event in atherogenesis. Oxidative stress induces cell proliferation, hypertrophy,

apoptosis and inflammation through activation of various signaling cascades, redox-sensitive transcriptional factors and expression of pro-inflammatory phenotype (Higashi et al., 2009). ECs dysfunction has been shown to be associated with an increase of ROS in atherosclerotic animal models and in human subjects with atherosclerosis (Dai, D.Z. & Dai, Y., 2010; Davies et al., 2010; Higashi et al., 2009). Moreover, in APOE-deficient mice, a widely used animal model of atherosclerosis (Xu, 2009; Zhang, S.H. et al., 1992), studies have demonstrated that aged-ECs are more sensitive to apoptosis than younger ones. ECs in the areas of the artery resistant to atherosclerosis have a life span of about 12 months, whereas cells at lesion-prone sites live for few weeks and even shorter in aged animals (Xu, 2009).

3.1 Endothelial cell-factors

The vascular endothelium is nowadays considered to be a paracrine organ responsible for the secretion of several substances exerting atherogenic effects. The reduced bioavailability of NO as an indirect result of the effects of those factors, leads to atherosclerosis and its clinical manifestations (Muller & Morawietz, 2009; Tousoulis et al., 2010). Under normal conditions, ECs constantly produce a number of vasoactive and trophic substances that control inflammation, VSMC growth, vasomotion, platelet function and plasmatic coagulation (Barton & Haudenschild, 2001; Traupe et al., 2003).

Normal vascular activity is essential for maintaining normal function of organs, dependent on a balance of vasoconstrictive and vasodilative substances derived from the endothelium, which mainly include NO to dilate and endothelin-1 (ET-1) to constrict the cells of tunica media. Furthermore, ECs activated by ROS can regulate vascular function via the release of inflammatory mediators, such as intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule (VCAM-1), MCP-1, ILs, angiotensin-II (A-II), TNFα, NF-kB and E- and P-selectin, or the release of haemostatic regulators, such as von Willebrand factor, tissue factor inhibitor and plasminogen activator, fibrinogen and NO (Sima et al., 2009; Vanhoutte, 2009).

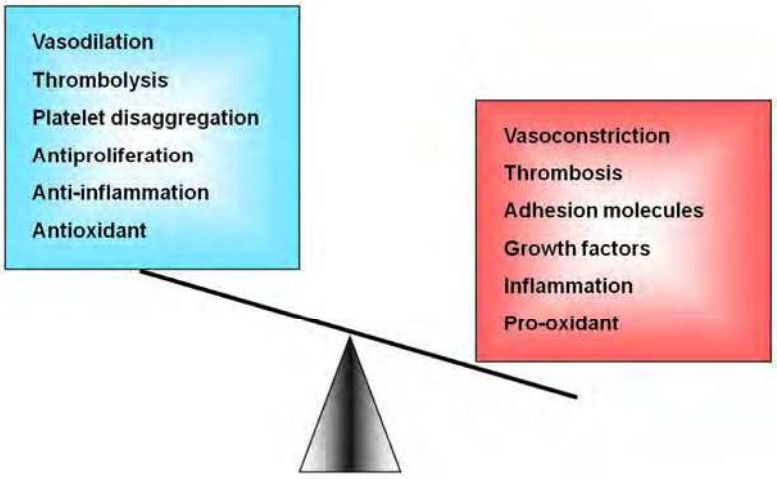

Fig. 5. Regulatory functions of the endothelium maintaining the equilibrium between antiatherogenic and atherogenic properties.

The purpose of the following paragraphs will be to provide a brief description and characterization of the main EC-factors that are synthesize and secrete after ROS stimulus during endothelial athero-susceptibility.

3.1.1 Angiotensin-II

A-II, a causal factor to the dysfunction of vascular endothelium, adversely stimulates the activity of the cardiovascular system (Dai, D.Z. & Dai, Y., 2010). A-II increases blood pressure by vasoconstriction and sodium and fluid retention and produces overt oxidative stress resultant from the activation of NADPH oxidase, a source of ROS in blood vessels, that promotes endothelial dysfunction, inducing cytokines, chemokines and adhesion molecules secretion and contributes to vascular remodeling (Dai, D.Z. & Dai, Y., 2010; Ferrario, 2009; Partigulova & Naumov, 2010). The A-II effects on gene expression are mediated, at least in part, through the cytoplasmic NF-kB transcription factor. Through these actions, A-II augments vascular inflammation, induces EC dysfunctions and, in so doing, enhances the atherogenic process (Sprague & Khalil, 2009).

3.1.2 Endothelial nitric oxide synthase

Endothelium-derived NO, formed by eNOS, (isoform 3 of NO shynthase) is known as a potent vasodilator (Barton, 2010). eNOS is also the master gene regulator used by ECs to orchestrate their own phenotype, function and survival. eNOS is modulated by shear stress (Rodella et al., 2010, a) and agonists acting on cell surface receptors; its activity is dependent on many mechanisms, including substrate availability, phosphorylation, Ca^{2+} flux and protein–protein interactions (Andrews et al., 2010).

With age, a number of changes occur in the cardiovascular system that can be considered pro-atherogenic (Barton, 2010). It is widely accepted that the most important mechanism leading to endothelial dysfunction is the reduced bioavailability of NO; so the decreased bioavailability of NO is consequently regarded a critical precursor to the development of atherosclerotic plaque and has been considered as one of the factors contributing to the higher incidence of atherosclerosis, arterial hypertension and renal disease in aged individuals (Barton, 2005). Together with its role as a vasodilator, NO impedes processes that are vital for atherosclerotic progression, including vasoconstriction, VSMCs proliferation and monocyte adhesion (Napoli et al., 2006). Furthermore, with atherosclerotic conditions, eNOS can become dysfunctional as it uncouples from its dimeric state to a monomeric state, in which it is able to produce superoxide anions rather than NO (Andrews et al., 2010; Vàsquez-Vivar et al., 1998).

3.1.3 Endothelin-1

Endothelins are EC-derived vasoactive peptides. Since its discovery, ET-1 has been demonstrated as one of the most potent known vasoconstrictors (Barton, 2010). ET-1 is synthesized in bulk by ECs and VSMCs (Rodella et al., 2010,a) as well as by macrophages, cardiomiocytes, neurons, renal medulla and Kupffer cells (Piechota et al., 2010). Factors that stimulate the release of ET-1 include endotoxins, TNFα, IL-1, adrenaline, insulin, thrombin and A-II. ROS are involved in the modulation and activation of ET-1 that induced various signaling pathways; in fact, during the inflammation process, atherosclerosis and hypertension there are elevated levels of ET-1 (Piechota et al., 2010; Skalska et al., 2009; Teplyakov, 2004).

3.1.4 Tumor necrosis factor α

TNFα is crucially involved in the pathogenesis and progression of atherosclerosis, myocardial ischemia/reperfusion injury and heart failure. The TNFα-mediated vascular dysfunction involves alterations in EC metabolism and function, platelet aggregation, EC-blood cell interaction, VSMC function and proliferation (McKellar et al., 2009). It increases the expression of many pro-inflammatory, pro-coagulant, proliferative and pro-apoptotic genes involved in initiation and progression of atherosclerosis (Bergh et al., 2009). TNFα induces the rapid expression of cellular adhesion molecules (CAMs), such as VCAM-1 and ICAM-1, and E-selectin at the endothelial surface (Chandrasekharan et al., 2007; Kleinbongard et al., 2010). Endothelial dysfunction associated with TNF*a* during atherogenesis is linked to an excess in production of ROS and a decrease in NO bioavailability. The production of ROS can stimulate a cytokine cascade through NF-κB-induced transcriptional events, which then induce the expression of TNFα (Zhang, H. et al., 2009).

3.1.5 Cellular adhesion molecules (ICAM-1 and VCAM-1)

When ECs undergo inflammatory activation, an increase in the expression of CAMs promotes the adherence of inflammatory cells (monocytes, neutrophils, lymphocytes and macrophages) and the recruitment of additional cytokines, growth factors and MMPs into the vascular wall (Sprague & Khalil, 2009). ICAM-1 and VCAM1 are immunoglobulin-like CAMs expressed by several cell types including ECs and leukocytes. They are present in atherosclerotic lesions during their progression, because they are involved in the transendothelial migration of leukocytes, lymphocytes and antigen presenting cells to sites of inflammation (Blankenberg et al., 2001; Ho et al., 2008; Lawson & Wolf, 2009; Rodella et al., 2010,b). Nevertheless their pathological role remain still uncertain. An important stimulus for CAMs expression is the fluid shear stress, which exerts both pro-inflammatory and protective effects, depending on the type of shear.

3.2 Shear stress

As the regulator of vascular tone, ECs are highly sensitive to different types of shear stress caused by the complex structure of artery geometry. It is clearly observed that atherogenesis generally occurs at curved or branching points with disturbed flow. Endothelium in the regions of flow disturbances near arterial branches, bifurcations and curvatures shows an athero-prone phenotype, while laminar flow regions exhibit an athero-protective phenotype (Bai et al., 2010; Traub & Berk, 1998). When endothelial monolayer is stimulated by laminar flow, rapidly cellular responses occur, included opening of ion channels, release of vasoactive NO and activation of transcription factors and cell cycle regulators (Foteinos et al., 2008). In particular, laminar flow induces NO production through both the transcriptional up-regulation of eNOS gene expression and the posttranslational modification of eNOS protein (Jin et al., 2003; Xu, 2009). Compared with ECs under laminar flow, cells at disturbed flow show an atherogenic phenotype as alterated alignment, deformation of luminal ECs surface, accelerated proliferation and apoptosis (Bai et al., 2010; Zeng et al., 2009), higher permeability, immunoinflammation responses and more athero-prone gene expression which are proportional to risk factor severity (Foteinos et al., 2008; Xu, 2000). Oscillatory shear stress leads to continuous O^{2-}

production in an NADPH-oxidase-dependent manner, resulting in NF-kB-mediated monocyte adhesion.
NF-kB is an inducible transcription factor present at increased levels in the thickened intima-media of atherosclerotic lesions, whereas little or no activated NF-kB has been detected in healthy vessels (Andrews et al., 2010; Rodella et al., 2010,b). The NF-kB pathway have been implicated in athero-susceptibility for more than a decade. NF-kB is normally held inactive in the cytosol as a complex with IkB, a family of inhibitors of NF-kB. Oxidative stress by ROS production induces IkB degradation, releases of NFkB for translocation to the nucleus where it regulates pro-inflammatory genes (Davies et al., 2010). Several pro-inflammatory cytokines and growth factors found in atherosclerotic lesions, such as TNFα, ILs, MCP-1 and tissue factors, activate NF-kB signaling pathway in cultured ECs (Pennathur & Heinecke, 2007). NF-κB plays a central role in the development of inflammation through further regulation of genes encoding pro-inflammatory cytokines, CAMs, chemokines, growth factors and inducible enzymes (Andrews et al., 2010; Sprague & Khalil, 2009).

3.3 EC-foam cells

The formation of foam cells as a result of the lipid loading in ECs is a late event in atherosclerosis. Since the atherogenesis process is gradual, it is known that plasma hypercholesterolemia is associated with increased transcytosis of lipoproteins (Lps), leading to their accumulation within the ECs. At this location, Lps interact with proteoglycans and other matrix proteins and carry on their conversion to oxidatively modified and reassembled Lps (MLps). MLps have been identified in early intimal thickenings of human aorta and in the late atheroma (Sima et al., 2009; Tirziu et al., 1995).
It is known that, in the initial stage of atherogenesis, upon the accumulation and retention of MLp within intima, the EC lining the plaque take up MLp, which are either degraded within the cell or exocytosed into the lumen; in time, the non-regulated uptake of MLp by the EC-scavenger receptor is overwhelmed, leading to the accumulation of numerous large lipid droplets within the ECs. Concurrently, the EC shifts to a secretory phenotype, characterized by an increased number of biosynthetic organelles that correlates with the appearance of a multilayer, hyperplastic basal lamina in meshes of which MLp in accumulate large numbers. These insults lead to a dysfunctional endothelium and inflammatory process in which the EC-derived foam cells express more of new CAM and synthesize EC-factors that attract and induce migration of plasma inflammatory cells, such as monocytes and T lymphocytes to the subendothelium (Simionescu & Antohe, 2006); however, ECs maintain some of their specific attributes, such as Weibel-Palade bodies, intercellular junctions and caveolae (Sima et al., 2009). Infiltration of atherogenic Lps, monocytes and T lymphocytes within the subendothelium start the atherogenetic process both in animal models and in humans (Lawson & Wolf, 2009; Simionescu & Antohe, 2006; Williams & Tabas, 2005). In late stages of atherosclerosis, all cellular components of the plaque, ECs, VSMCs and macrophages, accumulate considerable number of lipid droplets and exhibit the foam cell characteristics (Sima et al., 2009). In the subendothelium, the monocytes become macrophage-derived foam cells, which release cytokines and factors that, within the oxidative stress process, change the cross-talk between ECs and the neighbouring VSMCs and induce migration of VSMCs from media to the developing neointima (Lawson & Wolf, 2009; Simionescu & Antohe, 2006).

4. The role of vascular smooth muscle cells in atherosclerosis

VSMCs are important actors in the pathogenesis of atherosclerosis. The classical "*response to injury*" hypothesis of atherosclerosis suggests that one of the major events in the development of this pathology is the intimal thickening caused by hyperplasia and migration of VSMC in the tunica intima (Ross & Glomset, 1973): the combined action of growth factors, proteolytic agents, and ECM proteins, produced by a dysfunctional endothelium and/or inflammatory cells, induces proliferation and migration of VSMCs from the tunica media into the intima (Clowes et al., 1983; Hao et al., 2003). Finally, progression of atherosclerotic lesions in the intima is characterized by the accumulation of alternating layers of dedifferentiated VSMCs and lipid-laden macrophages (Sobue et al., 1999). This model focuses on the central role of activated and proliferating VSMCs that are histologically observed in the early and late stages of atherosclerosis, thus being a key event in atherosclerosis (Dzau et al., 2002; Owens, 1995). Because of their involvement in atherosclerosis, intimal VSMCs, their origin and the mechanisms that regulate their phenotype have been the subject of numerous studies and much debate over recent years.

4.1 Origin of intimal VSMCs in atherosclerosis

4.1.1 Phenotypic modulation of VSMCs

The long-standing dogma in the field has been that the majority of intimal VSMCs are derived from preexisting mature medial VSMCs that undergo phenotypic modulation on moving from the media to the intima (Owens et al., 2004). This hypothesis, proposed for the first time by Chamley-Campbell and colleagues (Chamley-Campbell et al., 1979) arose from a limited number of studies showing that in primary human cell cultures derived from different sources (e.g. medial cells or cells derived from atherosclerotic plaques) stable differences in phenotype could be identified. This dogma implies the potential for marked plasticity of the VSMC phenotype, with the ultimate phenotype being determined by a variety of extracellular stimuli (Bochaton-Piallat et al., 1996): numerous studies of cells cultured from different species have demonstrated that cytokines, matrix components, and mechanical stimuli can influence VSMC phenotype and behavior (Shanahan et al., 1993; Topouzis & Majesky, 1996).

VSMCs are the predominant cellular elements of the medial layer of the vascular wall, essential for good performance of the vasculature. VSMCs perform many different functions in maintaining vessel's health (Rensen et al., 2007). The VSMC is the only cell populating the normal vascular media, wherein it is uniquely responsible for maintaining vascular tone and hemodynamic stability: it is a highly specialized cell whose principal function is vasoconstriction and dilation in response to normal or pharmacologic stimuli to regulate blood vessel tone, blood pressure, and blood flow (Rzucidlo et al., 2007). Moreover, except in unusual circumstances when the adventitia may be involved, the VSMC is also the only vascular cell capable of repairing the injured vessel wall by migrating, proliferating, and elaborating an appropriate ECM. It is therefore equally essential that, when it is necessary, the VSMC can also adopt a phenotype capable of these synthetic functions (Shanahan & Weissberg, 1998). So, it is important that VSMCs retain remarkable plasticity and can undergo rather intense and reversible changes in phenotype in response to changes in local environmental cues, particularly under the influence of growth factors (Li, S. et al., 1999; Owens, 1995). In the pathogenesis of atherosclerotic lesions it is now accepted that VSMC can display at least two different phenotypes, the first characteristic of the media and the

second typical of the cells invading the intima (Shanahan & Weissberg, 1999). These phenotypes are also seen *in vitro*: an elongated spindle-shaped phenotype, with the classic ''hill-and-valley'' growth pattern typical of cultured contractile normal medial VSMCs and an epithelioid or rhomboid phenotype, with cells growing in a monolayer with a cobblestone morphology at confluence typical of the cells from neointima (Hao et al., 2003). In the medial layer of a mature blood vessel, VSMCs exhibit a low rate of proliferation, low synthetic activity and ECM proteins secretion, and express a unique repertoire of contractile proteins (e.g. intracellular myofilaments bundles are abundant), ion channels, and signalling molecules required for the cell's contractile function that is clearly unique compared with any other cell type (Rzucidlo et al., 2007). The dense body, the dense membrane and myofibrils (composed of thin filaments and myosin thick filaments) are well developed in differentiated VSMCs, whereas organelles (e.g. rough endoplasmic reticulum (RER), Golgi and free ribosomes) are few in number (Owens, 1995). This "contractile" state (referred also as "differentiated phenotype"), is required for the VSMC to perform its primary function. The gene expression pattern in end-differentiated VSMCs is well characterized and comprised a number of proteins involved in contraction, membrane-skeletal markers specific to smooth muscle and cell adhesion molecules and their receptors (integrins), which are important either as a structural component of the contractile apparatus or as a regulator of contraction (Owens, 1995; Rensen et al., 2007). Their expressions are regulated at the gene levels, such as at transcription and splicing: caldesmon, smooth muscle myosin heavy chain (SMM-HC), α-smooth muscle actin (α-SMA), h-caldesmon, calponin, SM22, α- and β-tropomyosins and α1 integrin genes are transcriptionally regulated; transcription of these genes (except for the α-smooth muscle actin gene) is upregulated in differentiated VSMCs, but is downregulated in dedifferentiated VSMCs (Stintzing et al., 2009). It's important to note that, although α-SMA is permanently expressed in VSMCs, it is more abundant in contractile VSMCs than in synthetic VSMCs (Lemire et al., 1994). Isoform changes of caldesmon, α-tropomyosin, vinculin/metavinculin, and SMM-HC are instead regulated by alternative splicing in a VSMC phenotype-dependent manner (Sobue et al., 1999). At present, the two marker proteins that provide the best definition of a mature contractile VSMC phenotype are SM-MHC and smoothelin. SM-MHC expression has never been detected in non-VSMCs *in vivo*, and is the only marker protein that is also VSMCs-specific during embryogenesis (Miano et al., 1994). Smoothelin complements SM-MHC as a contractile VSMC marker in that it appears to be more sensitive.

On the contrary, intimal VSMCs associated with vascular disease (as well as VSMCs involved in blood vessel formation) are phenotypically distinct from their medial counterparts (Campbell, G.R. & Campbell, J.H., 1985; Mosse et al., 1985): they resemble immature and show a typical "synthetic" state (referred also as "dedifferentiated phenotype"), characterized by an increased rate of proliferation, migration and ECM protein synthesis. Several studies by Aikawa and coworkers (Aikawa et al., 1997, 1998) demonstrated that intimal VSMCs show a synthetic phenotype including: *1*) increased DNA synthesis and expression of proliferation markers and cyclins (Gordon et al., 1990); *2*) decreased expression of smooth muscle-specific contractile markers (Layne et al., 2002); *3*) alterations in calcium handling and contractility (Hill et al., 2001); *4*) alterations in cell ultrastructure, including a general loss of myofilaments, which is replaced largely by synthetic organelles such as RER and large Golgi complex (Sobue et al., 1999), supporting its function in production and secretion of ECM components that, leading to intimal

thickening and fibrosis of the vascular wall, may contribute to lesion development and/or stability (Schwartz et al., 1986, 1995). The preceding studies have been extended by Geary and colleagues (Geary et al., 2002), who completed microarray-based profiling of gene expression patterns of SMCs in the neointima. A total of 147 genes were differentially expressed in neointimal VSMCs versus normal aorta VSMCs, most genes underscoring the importance of matrix production during neointimal formation. Therefore, these VSMCs assume the proliferative activity in response to mitogens, while lose contractile ability. Markers that are upregulated in the synthetic phenotype are rare. SMemb/non-muscle myosin heavy chain isoform B (MHC-B) represents a suitable synthetic VSMCs marker, since this protein is quickly and markedly upregulated in proliferating VSMCs (Neuville et al., 1997). At last, an interesting correlation has been demonstrated, albeit occasionally, between dedifferentiated VSMC phenotype and increased LDL uptake (Thyberg, 2002) or decreased HDL binding sites (Dusserre et al., 1994). Nevertheless, the role of LDL and HDL processes in atheromatous plaque formation with respect to VSMC heterogeneity should be further investigated.

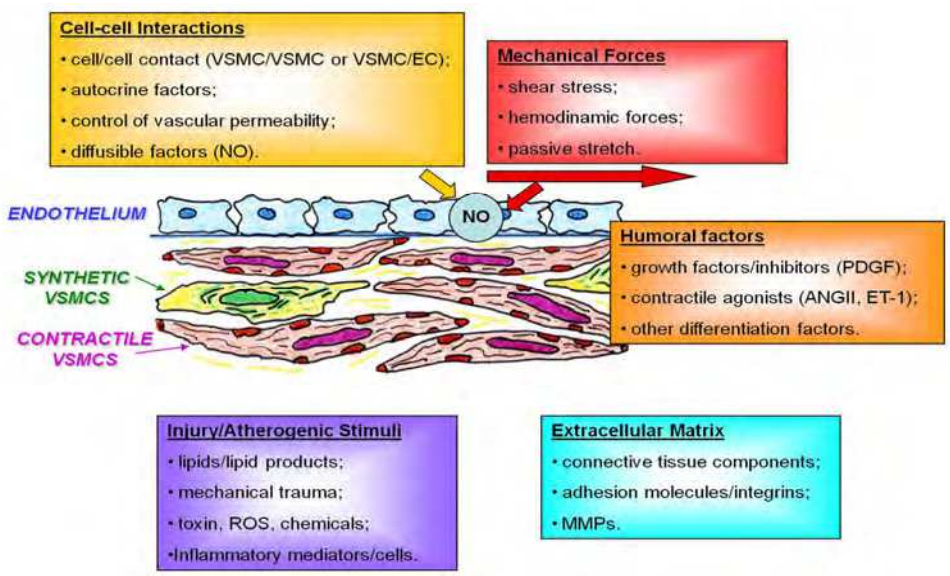

Fig. 6. Factors involved in VSMCs development, differentiation and phenotypic modulation

However, it is now recognized that a simple two-state model, based on "contractile" and "synthetic" states only, is inadequate to explain the diverse range of phenotypes that can be exhibited by the VSMCs under different physiological and pathological circumstances (Owens et al., 2004). In particular, the environmental cues that exist within atherosclerotic lesions are without doubts very different from those that exist within a normal healthy blood vessel and these change at different stages of lesion development and progression and thereby are likely to contribute to continued phenotypic switching of VSMC within the lesion. So, an heterogeneity of VSMC phenotype, ranging from contractile to synthetic, which represent the two ends of a spectrum of VSMCs with intermediate phenotypes, is

nowadays considered. Not surprisingly, as the repertoire of VSMC markers has expanded, the picture that has emerged is that there is likely a wide spectrum of possible VSMC phenotypes that might exist such that it may be very artificial to assign cells to distinct subcategories. So the distinction between "contractile" and "synthetic" state of the VSMC become very difficult. The complexity of different phenotypes that may be manifested by VSMC is clearly evident not only between VSMCs of different vessels or among VSMCs within the same vessel, but there is very clear evidence that the properties of the VSMCs vary also at different stages of atherosclerosis, within different lesion types, and between VSMCs located in different regions within a given lesion (Owens et al., 2004).

4.1.2 Monoclonality of atheromatous lesion and heterogeneity of proliferating VSMCs

Alternative to the predominant hypothesis that all VSMCs of the media can undergo phenotypic modulation, is the concept that a predisposed VSMCs subpopulation is responsible for the production of intimal thickening. This possibility has been raised on the basis of original work by Benditt and Benditt (Benditt, E.P. & Benditt, J.M., 1973) who reported that VSMC accumulation in the atheromatous plaques is monoclonal or, at least, oligoclonal (Chung et al., 1998), implying that only a small number of "immature" cells in the vessels media and/or adventitia undergo proliferation (Holifield et al., 1996). More recent studies have questioned the origin of VSMCs comprising atherosclerosis and neointima formation. Intimal VSMCs have been proposed to originate from diverse sources, including fibroblasts of the adventitia (Zalewski et al., 2002), ECs (Gittenberger-de Groot et al., 1999) and/or circulating bone marrow–derived cells (Hillebrands et al., 2003). Whereas the gene expression pattern of differentiated VSMC is pretty well characterized (Shanahan & Weissberg, 1999), many *in vivo* and *in vitro* studies dealing with proliferating VSMC showed heterogeneous cell marker expressions of multilineage differentiation (Tintut et al., 2003). A possible explanation of the heterogeneity of VSMCs in adult vessels can be found in embryologic vascular development (Gittenberger-de Groot et al., 1999): interestingly, similar to atherosclerosis, processes of multilineage differentiation with transition states could be observed during vascular development (Slomp et al., 1997). During vasculogenesis, VSMCs originate from different sources via transdifferentiation (Liu et al., 2004) (a highly conserved phenomenon of transdifferentiation is proved by a stable cytokeratins expression in atherosclerotic lesions as well as it happens during development (Neureiter et al., 2005)) depending on the vessel type, including mesoderm, neurectoderm, epicardium (for coronary arteries) and, more rarely, endothelium (Orlandi & Bennett, 2010). It is thus possible that the various VSMC phenotypes can arise from distinct lineages. Another possibility is that local VSMC of the contractile phenotype re-obtain the embryonic potential of proliferation and migration (Bar et al., 2002) via transdifferentiation and dedifferentiation processes as a response to injury. Looking at atherosclerosis and VSMC, there is a lot of evidence that VSMC progenitor cells are essentially involved in the progression of atherosclerosis (Roberts et al., 2005).

The origin of such VSMC progenitor cells is under debate. VSMC progenitor cells have been identified in the bone marrow (multipotent vascular stem cell progenitors and mesenchymal stem cells), in the circulation (circulating VSMC progenitor cells), in the vessel wall (resident VSMC progenitor cells and mesangioblasts) and various extravascular sites (extravascular, non-bone marrow progenitor cells) (Orlandi & Bennett, 2010).

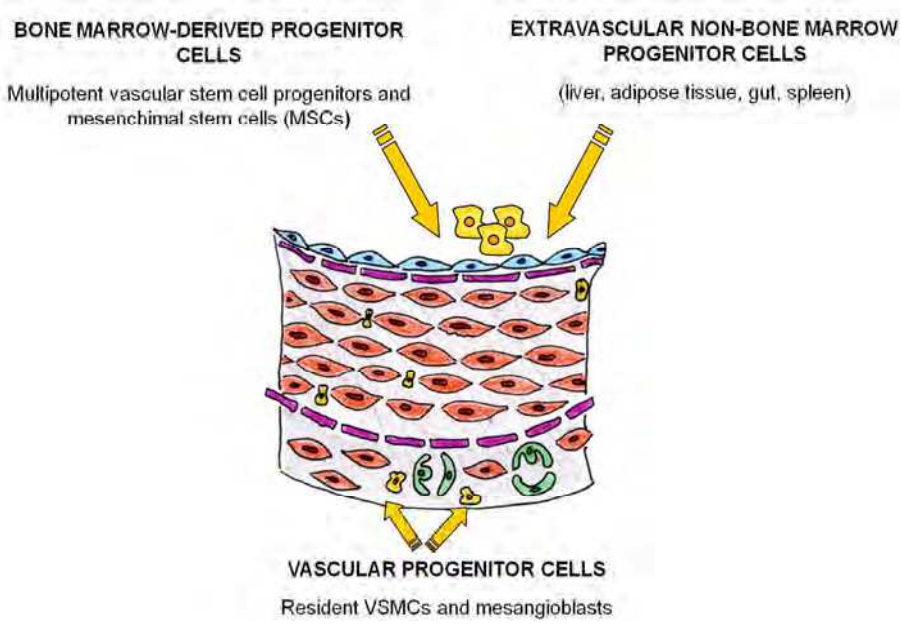

Fig. 7. Different origins of VSMCs progenitor cells.

4.1.2.1 Bone marrow-derived VSMCs

Several studies have suggested that circulating bone marrow-derived cells contribute to neointima formation: one possibility is that circulating smooth muscle precursor cells of myeloid or hematopoietic lineage relocate from the blood into the neointima following vascular injury (Metharom et al., 2008) and start to proliferate giving rise to cells that express at least some properties of VSMCs (Simper et al., 2002).
Other studies, on the other hand, report no evidence for a contribution of bone marrow derived VSMCs in the neointimal layer (Hu et al., 2002; Li, J. et al., 2001). Alternatively, these circulating cells may fuse with resident VSMCs and thus show co-localization of VSMC markers and bone marrow lineage markers, although to date, no direct evidence for cell fusion in the vasculature has been shown (Owens et al., 2004).

4.1.2.2 Resident VSMC progenitor cells and mesangioblasts

Inside normal vessel walls the existence of resident progenitor cells (expressing stem cell antigens) capable of contributing to neointima formation has been recently shown (Orlandi et al., 2008; Torsney et al., 2007): the number of these resident VSMCs progenitors has been shown to increase in atherosclerotic lesions (Torsney et al., 2007). These progenitor cells are different from marrow-derived smooth muscle progenitor cells, since they lack the ability to differentiate into erythroid, lymphoid, or myeloid tissue (Jackson et al., 1999). Subsequent studies examining telomere loss indicate that fibrous cap VSMCs have undergone more population doublings than cells in the normal media (Matthews et al., 2006), suggesting the existence of a resident arterial subpopulation predisposed to clonally contribute to arterial healing in response to injury (Hirschi & Majesky, 2004), so that plaques arise by selective expansion of a preexisting 'patch' of progenitor cells.

Unfortunately, against this theory, there is very limited evidence for the presence of vessel wall stem cells in human vessels. A population of $CD34^+/CD31^-$ cells has been identified in the space between the media and adventitia of large and medium-sized human arteries and veins (Pasquinelli et al., 2007), but the capacity of these cells to give rise to VSMCs was low (Zengin et al., 2006). Few other studies showed that the adventitial layer potentially harbours a population of stem cells that can also contribute to vascular remodelling. In particular, Hu and colleagues demonstrated that abundant progenitor cells in the adventitia can differentiate in VSMCs (Hu et al., 2004).
Moreover, satellite-like cells named 'mesoangioblasts' express both myogenic and EC markers (Drake et al., 1997), which can give rise to both hematopoietic and endothelial progenies (Cossu & Bianco, 2003). Gene expression profiles reveal that mesoangioblasts express genes belonging to developmental signaling pathways (such as β-catenin/Wnt signaling pathway) and are able to differentiate very efficiently into VSMCs (Tagliafico et al., 2004).
In summary, there is evidence for several distinct resident progenitor cells in different layers of the normal adult arterial wall capable of proliferating and differentiating into VSMCs. What has not yet been established is how many of these cells contribute to formation of vascular lesions and whether clonality reflects selective proliferation of one or more of these populations.

4.2 VSMCs: Friend or foe in atherosclerosis?

It is important to note that the exact role of VSMCs, in the progression of atherosclerosis is not clear. The functional role of VSMCs likewise is likely to vary depending on the stage of the disease. For example, at the early onset of atherosclerosis, these cells presumably plays a maladaptive role, because of their involvement in neointima formation (Rodella et al., 2011): mobilisation of these cells would therefore be predicted to promote, as a "foe", vascular disease (van Oostrom et al., 2009). On the other hand, over recent years, there has been an increasing recognition of the role played by intimal VSMCs in the formation and maintaining of a protective fibrous cap over the atherosclerotic plaque, desirable for plaque stability in the advanced atherosclerotic process (Weissberg et al., 1996). In particular, IFN-γ released by activated macrophages induces collagen synthesis by VSMCs, which is important for the stabilization of the fibrous cap (Shah et al., 1995). Moreover, injection of smooth muscle progenitor cells in a mouse model of advanced atherosclerosis reduced the progression of early atherosclerotic plaques (Zoll et al., 2008), confirming the potential benefit of VSMCs at advanced stages of atherosclerosis. Therefore, VSMCs could be beneficial in atherogenesis as a factor promoting plaque stability and can thus be considered a "friend" in vascular disease (van Oostrom et al., 2009).
Since the VSMC is the only cell capable of synthesizing the fibrous cap, failure of this vascular repair response leads to weakening of the cap and plaque rupture, with potentially fatal consequences (Weissberg et al., 1996). In diseased tissue many factors are present that substantially alter the normal balance of proliferation and apoptosis, and the apoptosis may predominate (Bennett, 2002). In particular, in plaque VSMCs an elevated level of spontaneous apoptosis and enhanced susceptibility to apoptosis induced by ROS (Li, W.G. et al., 2000) has been recently described both *in vivo* and *in vitro* (Ross, 1999). Apoptosis of VSMCs, bringing a plaque with reduced number of VSMCs, could participate in the rupture of the stability of the plaque (Rudijanto, 2007). Rupture of atherosclerotic plaques is

associated with a thinning of VSMC-rich fibrous cap overlying the core (atrophic fibrous cap lesion), due to rapid replicative senescence and apoptosis of VSMCs (Schwartz et al., 2000). Rupture occurs particularly at the plaque shoulders, which exhibits lack of VSMCs and the presence of inflammatory cells (Newby et al., 1999). So, VSMCs may later contribute to plaque destabilization through apoptosis and/or activation of various protease cascades (Galis & Khatri, 2002).

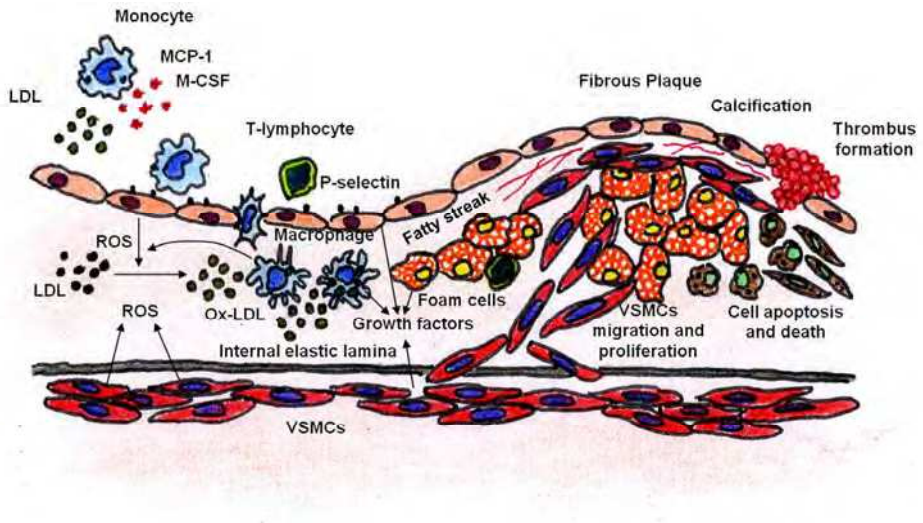

Fig. 8. Involvement of VSMCs apoptosis in fibrous plaque rupture.

However, detailed studies demonstrating whether VSMC progenitors either protect or promote vessel disease are needed before cell-based or pharmacological approaches aimed at regulating progenitor cell trafficking can be recommended.

4.3 VSMCs can auto-regulate their replication/migration

The contemporary paradigm explaining smooth muscle replication in the vessel wall is that dysfunctional endothelium and/or inflammatory cells produce growth factors and ECM proteins that can induce replication and migration of VSMCs from the media to the intima (van Oostrom et al., 2009). In his "*response-to injury*" hypothesis, Ross proposed that VSMCs in the wall normally exist in a quiescent state, but, when the endothelium is injured, platelets release factors that stimulate VSMCs movement into and replication within the arterial intima (Ross, 1981-1982).

Growth factors have been known to influence the differentiated state of VSMCs (Willis et al., 2004). An interesting possibility is that smooth muscle replication may be controlled by factors intrinsic to the vessel wall. One possibility comes from evidence that normal endothelium contains inhibitors of smooth muscle proliferation (Haudenschild & Schwartz, 1979). The principal factor involved in VSMCs replication is the platelet derived growth factor (PDGF), which is a potent VSMC mitogen linked to vascular homeostasis and atherogenesis (Majesky et al., 1992). This peptide not only is mitogenic for VSMCs, but is chemotactic as well (Schwartz et al., 1986): the data on PDGF and its receptor subunits

suggest, infact, a role in migration/localization of primordial VSMCs to the endothelium. This growth factor consists of two chain types, A and B, giving rise to three different PDGF subtypes (AA, AB, BB): PDGF-BB and -AB are known VMSCs chemoattractans, whereas PDGF-AA is associated with inhibition of chemotaxis (Zachary et al., 1999). PDGF binds to specific dimeric receptors (α and β) found on smooth muscle cells (Bowen-Pope & Ross, 1982) where initiates a series of events leading to DNA synthesis: receptor α can bind all PDGF subtypes, while receptor β binds only subtypes -AB and -BB. VSMCs have been determined to upregulate expression of receptor β in response to vascular injury, inducing their chemotaxis; at the same time, these cells are able to increase the PDGF-AA, acting as a paracrine or autocrine regulator of their chemotaxis. This represents the first described autoregulation pathway of VSMCs on their own proliferation/migration (Willis et al., 2004).
The second known requirement for cell cycle progression is availability of insulin-like growth factor (IGF-1), a co-factor that VSMCs require for completion of the cell cycle following stimulation with PDGF (Clemmons, 1984). Perhaps more surprising is that, as reported above, VSMCs may be able to stimulate their own growth by synthesis of both PDGF and IGF-1 (PDGF is able to stimulate smooth muscle cells to produce IGF-1).
Moreover, those VSMCs that, once migrated into the intima, retained the ability to produce mitogen, due to their dedifferentiated state (Schwartz et al., 1986), are able to sustain proliferation also after the initial stimulation of platelet and PDGF release during vascular injury. Selection of such a proliferogenic subpopulation could account for both the monoclonal phenotype of chronic human atherosclerotic lesions (Gown & Benditt, E.P., 1982) and the suggestion that monoclonality arises gradually as the human lesion evolves (Lee et al., 1985). In summary, the emerging picture of growth control in arterial smooth muscle is a complex balance of forces. In addition to exogenous stimuli to cell growth, the vessel wall is capable of synthesis of endogenous growth inhibitors (including heparin sulfates, nitric oxide (NO), and transforming growth factor (TGF)-β) and growth stimulants (such as PDGF, IGF-1, ET-1, thrombin, FGF, IFNγ, and IL-1) (Berk, 2001).

5. Conclusions

Atherosclerosis and its associated complications remain the primary cause of death of the 21st century in humans. Recently it has been suggested that atherosclerosis is a multifactorial, multistep disease. Clinical and histopathological studies of atherosclerotic patient groups have identified inflammatory and oxidative stress-linked mechanisms as being pathogenetically important in atherosclerosis at every step from initiation to progression. Endothelial damage is also crucial for the progress of atherosclerosis and risk factors for atherosclerosis represent crucial factors associated with endothelial dysfunction. Studies have shown that patients with cardiovascular disease are characterized by impaired endothelial function, being vascular endothelium responsible for the secretion of several substances exerting proved anti-atherogenic effects. Finally, VSMCs are an important component of atherosclerotic plaques, responsible for promoting plaque stability in advanced lesions. In contrast, VSMC apoptosis has been implicated in a number of deleterious consequences of atherosclerosis, including plaque rupture, vessel remodelling, coagulation, inflammation and calcification. A better understanding of the pathogenesis of atherosclerosis will aid in for reducing mortality. An in-depth knowledge of the various pathogenic mechanisms involved in atherosclerosis can help in formulating preventive and therapeutic strategies and devising pharmaceutical and lifestyle modifications for reducing mortality.

6. Acknowledgments

The Authors want to thank Dr. Favero Gaia, Dr. Foglio Eleonora and Dr. Rossini Claudia for their excellent work in writing and improving this chapter and for their contribution in realizing the figures. Moreover, the Authors thank Peroni Michele for his help in editing this chapter.

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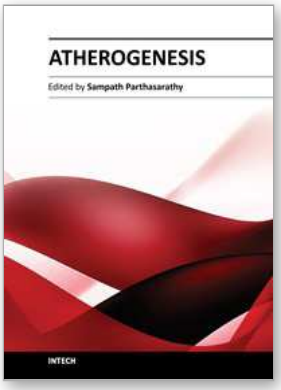

Atherogenesis

Edited by Prof. Sampath Parthasarathy

ISBN 978-953-307-992-9
Hard cover, 570 pages
Publisher InTech
Published online 11, January, 2012
Published in print edition January, 2012

This monograph will bring out the state-of-the-art advances in the dynamics of cholesterol transport and will address several important issues that pertain to oxidative stress and inflammation. The book is divided into three major sections. The book will offer insights into the roles of specific cytokines, inflammation, and oxidative stress in atherosclerosis and is intended for new researchers who are curious about atherosclerosis as well as for established senior researchers and clinicians who would be interested in novel findings that may link various aspects of the disease.

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