Chapter 3

Endothelial Cell Senescence in the Pathogenesis of Endothelial Dysfunction

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Additional information is available at the end of the chapter

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Abstract

Aging is the main risk factor for cardiovascular diseases (CVD), and senescence in endothelial cells seems to be an initial step in the cascade of events that will culminate with the development of these pathologies. In this chapter, we examine the pathophysiological mechanism(s) involved in endothelial senescence, leading to CVD as well as the biochemical and cellular pathways that may explain the activation and development of the process of endothelial senescence, and we discuss new hypotheses supported by experimental results which suggest that the senescent endothelial cell may induce a general process of vascular senescence. This process is probably induced either by soluble molecules secreted by these senescent cells and/or by intercellular signals transported in cellular vesicles that may be useful as biomarkers and as potential therapeutic targets in endothelial senescence.

Keywords: aging, biomarkers, cardiovascular disease, endothelium, microvesicles

1. Introduction

The term "cardiovascular diseases" (CVD) refers to a group of pathologies that share a common nexus, as they are preceded by process of damage and endothelial dysfunction. The imbalance of oxidative stress within the endothelium promotes the activation of cellular senescence processes, altering the biological functions of endothelial cells [1] and favoring CVD development. Indeed, chronologic aging or premature senescence (caused by pathologic environment) is significantly associated with CVD development [2].

Cellular senescence is an irreversible biological phenomenon triggered by potentially harmful stimuli which can damage the cell genome. During this process, the cell interrupts the division process, entering a state of cell cycle arrest and becoming quiescent. Senescence is a protective mechanism which affects the major part of the cells within the organism, including the vascular cells [3, 4]. It is considered indispensable to prevent tumor development, although turns to be pathologic when senescent cells extensively accumulate in tissues as a consequence of aging.

Cell senescence can be triggered prematurely due to aging-associated pathologies such as CVD or chronic kidney disease (CKD). In fact, several studies confirm that CKD patients manifest premature aging in several tissues, including those in the cardiovascular system [5]. This is partly explained because CKD patients show "classic" cardiovascular risk factors (age, lifestyle, left ventricular hypertrophy, dyslipidemia, hypertension and diabetes mellitus). Kidney failure leads to the accumulation of circulating uremic toxins in the blood of those patients, causing stress and damage to the endothelium and activating endothelial cells senescence. Furthermore, CKD patients often show subclinical chronic inflammation associated with an immunosenescence process, which seems to be induced by the uremic toxins and other factors [6]. The renal replacement therapies may have a significant role in this process, as they induce the activation of immunocompetent cells [7].

Taken together, these concepts show that blood circulating toxins cause endothelial cells to become senescent leading to the appearance of several CVD. For example, some studies have proved that, at least in atherosclerotic processes, the pathogenic basis by which the CVD is developed is endothelial senescence [8, 9]. When endothelial cells become senescent, their imbalanced functionality may lead to the loss of the vascular structure. Moreover, the senescent endothelium cannot regulate correctly the repairing and regenerative activity of endothelial progenitor cells (EPCs), which increases the harmful effect in the vascular bed [10]. It is easy to understand in this context that endothelial senescence acts as the first element in the development of CVD.

Recently, microvesicles (MVs) have been proposed as endothelial response elements that can take part both in damaging and repairing processes in the endothelium [10–12]. There is certain knowledge, yet scarce, about the mechanisms underlying the participation of MVs in endothelial homeostasis, although the implication of those MVs in endothelial senescence remains an unresolved question.

Therefore, to understand and characterize the mechanisms by which the senescent endothelial cells show an imbalanced functionality, it is necessary to identify early biomarkers and to design therapeutic targets for CVD.

2. Endothelial dysfunction as the first step in the development of vascular disease

Endothelial dysfunction is an earlier pathophysiologic stage in CVD development. Ross in 1976 published his theory of response to damage, where he hypothesized that the initial event in atherogenesis is the endothelial injury, followed by the proliferation of smooth muscle

cells [13]. Over the past years, this theory has been consolidated as endothelial damage is shown to be decisive in the promotion of vascular diseases. Indeed, diverse pharmacologic and dietetic interventions are intended to prevent the imbalance of the endothelial function, trying to interfere with the development of atherosclerosis and its clinical consequences [14, 15].

The endothelium is a thin monocellular layer that covers the inner surface of blood vessels, separating the circulating blood from the interstitial fluid [16]. The endothelium is not an inert organ, as it can respond to physical or chemical stimuli by liberating the adequate regulatory substance to keep the correct vasomotor equilibrium and homeostasis [17]. The endothelium acts as an autocrine, paracrine and endocrine gland. Endothelial cells produce vasodilating, antiproliferative, antithrombotic and antiadherent mediators, like nitric oxide (NO), prostacyclin, the endothelium-derived hyperpolarizing factor (EDHF) and the natriuretic peptide, type C (CNP). The actions of those molecules are compensated by the release of substances with the opposing effect, as endothelin 1, thromboxane A2, prostaglandin H2 and the superoxide anion. Thus, endothelium regulates the tone of the smooth muscle cells of the vessel wall, causing its relaxation or contraction and conditioning the vasodilation or vasoconstriction processes. Also, it regulates hemostasis by controlling the production of prothrombotic or antithrombotic molecules, as well as fibrinolytic and antifibrinolytic substances. Endothelium takes part in inflammatory and immune processes by regulating proliferation and cell migration, as well as adherence and leukocytes activation. It is capable of producing cytokines and adhesion molecules that regulate the inflammatory process, contributing to the defensive function of the organism by the activation of neutrophils and macrophages [18].

Cardiovascular risk factors provoke an oxidative stress which alter the function of the endothelial cells and provoke endothelial dysfunction by reducing the ability of the endothelium to maintain the homeostasis and concluding with the development of vascular diseases [19]. The term "endothelial dysfunction" has been used to define diverse syndromes which include a change of the endothelial phenotype from a "basal" to an active state. It is a complex disorder which includes alterations in the vasomotor and antithrombotic responses, in the vascular permeability, the leukocytes recruitment and the proliferation of endothelial cells [20, 21]. In the progress of endothelial dysfunction, the presence of pathologic conditions can contribute accelerating CVD development [22, 23].

Among the cardiovascular risk factors, the age arises as a critical factor. It is associated with damage and endothelial dysfunction, as well with atherosclerosis development which will lead to vascular pathologies [24]. Epidemiologic studies have demonstrated that aging is the most important risk factor for the development of CVD, mainly atherosclerotic [23]. During the gradual aging, the incidence and prevalence of atherothrombotic and coronary diseases and cerebrovascular accidents increases. For that reason, there must be a causal relationship between the age-associated changes and vascular damage. It has been demonstrated that, during aging, the vasculature of healthy subjects suffers several changes, as endothelial dysfunction [21], the arterial wall thickening and remodeling [25], angiogenesis alterations, incorrect vascular repair [26] and increased atherosclerosis prevalence [27]. The relationship between the development of these disorders and the aging process remain poorly understood, but it is possible that throughout the physiologic aging of the organisms some similar changes occur, comparable to those in the vascular diseases and sharing common cellular mechanisms.

3. Endothelial cellular senescence as pathophysiological mechanism of vascular pathology

One of the mechanisms that have been postulated as a possible pathophysiological participant is the cellular senescence of the endothelium. Cellular senescence is an irreversible process typical for all cells in which cells leave the cycle division as a consequence of the cellular damage associated with diseases [28] and aging [29]. Cell senescence processes appear to be involved in physiological processes of control such as cancer protection, biological developmental processes, tissue repair in aging situations and age-related disorders. Although their involvement in the aging process was postulated by Shay and Wright (Hayflick limit) [30], the absence of specific markers of senescence has hampered efforts to characterize senescent cells that accumulate *in vivo* in tissues and organs. Nowadays, the process of cell senescence is becoming better known due to the availability of new techniques to determine and quantify the senescent characteristics. In general, the main characteristic of the senescent phenotype is that cells decline in DNA replication until they cease to proliferate associated with the molecular changes of elements related to the cell cycle [31]. In general, senescent cells exhibit an upregulation and secretion of growth factors, proinflammatory cytokines, and also they release extracellular matrix-degrading proteins, the overall contribution constitutes the senescence-associated secretory phenotype (SASP) [32] and cells lose the ability to divide at the end of replicative lifespan and decrease their ability to migrate [33]. At a phenotypic level, senescent cells acquire the typical flattened and enlarged morphology [34] (**Figure 1**). Aforementioned

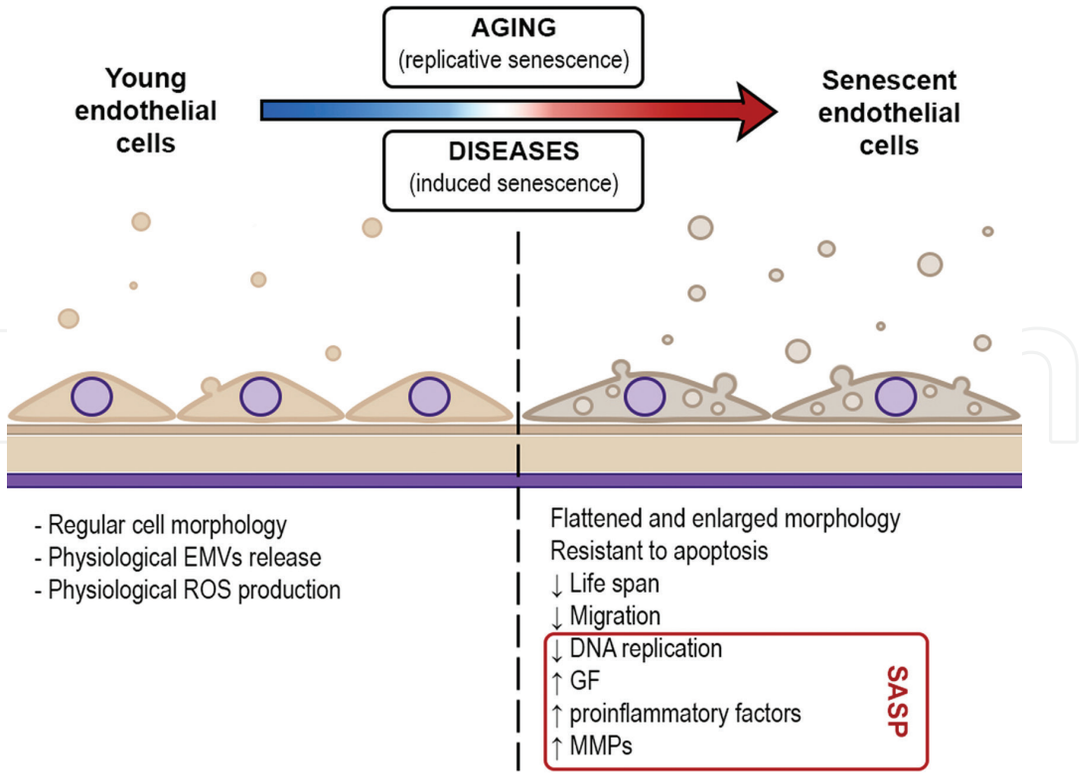

Figure 1. Mechanisms by which endothelial cells become senescent and their characteristics. GF, growth factors; MMPs, matrix metalloproteinases; SASP, senescence-associated secretory phenotype; EMVs, endothelial microvesicles; ROS, reactive oxidative species.

cells undergo distinctive phenotypic alterations, including profound chromatin and secretome changes, telomere shortening, genomic and epigenomic damage, unbalanced mitogenic signals and tumor-suppressor activation [28, 29]. Also, in human replicative senescence, telomere lengths decline with each cell cycle [35]. Most of these cells are resistant to some apoptosis signals, therefore, they become senescent [31]. Senescence and apoptosis are responses to cellular stress, and both are important in the activation of tumor suppressors [36], but senescence avoids the damage in the stressed cells. To date, some senescence markers have been described (**Table 1**) that are involved in cellular senescence, most of which participate in cell cycle control and DNA repair [31]. Further analysis has highlighted that many common

Characteristics	Markers	Regulation	Techniques	References
DNA replication (senescent cells decline in DNA replication)	BrdU	↓	Fluorescence microscope	[31]
	^{3}H-dT	↓	Incorporation of radioactivity	
	PCNA	↓	Immunostaining/Western blot	
	Ki-67	↓	Immunostaining/Western blot	
SA-β-gal activity (the SA-β-gal derives from the lysosomal β-galactosidase and reflects the increased lysosomal biogenesis)	X-gal substrate	↑	Light microscopy (production of blue precipitate)	[41, 42]
	C_{12}FDG (fluorogenic substrate)	↑	Fluorescence microscopy (production of green fluorogenic color)	
Cell cycle arrest proteins (early markers of DNA damage-induced senescence)	p16	↑	Western blot/immunostaining	[43–45]
	p21			[29, 46]
	p53			
	Cyclin D1			[38]
	Lamin B1	↓		[39]
SAHFs (reorganization of chromatin into discrete foci)	DNA dyes: DAPI	↑ Presence of certain heterochromatin-associated histone modifications	Fluorescence microscopy	[31, 47]
SDF (different DNA repair proteins)	γ-H2AX: marker of DNA double strand breaks and genomic instability	↑	Fluorescence microscopy/Western blot	[31]
	53BP1: protein associated with DNA damage	↑	Fluorescence microscopy	

BrdU, 5-bromodeoxyuridine; ^{3}H-dT, ^{3}H thymidine; PCNA, Proliferating cell nuclear antigen; SA-β-gal, Senescence-associated β-galactosidase; X-gal substrate, 5-bromo-4-chloro-3-indolyl-D-galactoside; C_{12}FDG, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside; SAHFs, senescence-associated heterochromatin foci; DAPI, 4′,6-diamidino-2-phenylindole; SDF, senescence-associated DNA damage foci; γ-H2AX; phosphorylated histone H2AX; 53BP1, p53-binding protein-1.

Table 1. Senescence markers.

cellular markers of senescence (upregulation of senescence-associated (SA)-β-galactosidase (gal) and p16) [29] are not robust and might overestimate the numbers of senescent cells that are present at low frequencies [37]. Thus, other cellular markers, such as cyclin D1 and lamin B1 [38, 39], are considered more reliable markers of senescence.

The use of all these elements to define senescent cells has provided convincing evidence that these senescent cells accumulate in tissues of humans, primates and rodents with advanced age, as well as in sites of tissue injury and remodeling. The most prominent feature of the senescent cells is a cell cycle arrest, which permanently withholds replication and the resistance to apoptosis. An important fact to note is that the cells with senescent characteristics are found in damaged tissues of patients with chronic diseases such as osteoarthritis, pulmonary fibrosis, atherosclerosis, Alzheimer's disease or CKD [40].

4. Chronic kidney disease, a model of chronic pathology that accelerates endothelial aging

CKD is known to promote cellular senescence and an accelerated aging. It is caused by the accumulation of toxins in the internal medium, and the consequence is the development of elderly associated pathologies, mainly CVD [48]. CKD-associated CVD show similar characteristics to the natural CVD in elderly, and for this reason, several authors propose that the biggest challenge in the treatment of CVD may be to understand why CKD promote the premature aging of the cardiovascular system [49].

Even though the progress in the last few years in the renal replacement therapy is substantial, the mortality of terminal CKD patients remains excessively high, with an incidence between 10 and 20-fold over the general population [50].

Uremic patients have higher rates of cardiovascular morbidity and mortality than would be predicted by Framingham risk factors [50–52]. However, the presence of those factors is not enough to explain the significant increment of the cardiovascular risk in those patients. CKD patients show additional factors associated with uremia that could explain this increased CVD risk [53]. The presence of microalbumin and uremic toxins in blood, hyperhomocysteinemia, anemia, the abnormal calcium/phosphate metabolism, parathyroid hormone (PTH) level alterations, the treatment with vitamin D derived substances, the volume overload, the electrolytic imbalance, oxidative stress, inflammation, malnutrition, thrombogenic factors and the imbalance of NO/endothelin are risk factors intrinsically associated to CKD [54]. The valuation and modulation of those factors are of high importance in CKD patients, as some are variable and the correct treatment may prevent the progression of the pathology.

In CKD patients, the endothelium is exposed to an additional stress because of the presence of factors related to the uremic state. This state can be modified depending on the conservative treatment or renal transplantation, but it has been demonstrated that it relies on a persistent microinflammatory state directly related to endothelial damage, partaking in atherosclerosis processes [7, 55, 56]. Under this hostile uremic-associated state, the endothelium loses its integrity. Some damage substances and molecules will be released as a reflection of the harmful stimuli [56, 57].

Among several inflammatory factors, the subpopulation of monocytes habitually augmented in elderly, increases in the peripheral blood. The contribution of monocytes in inflammation and the CVD development has been widely studied by several groups, including ours [58]. Peripheral blood monocytes show a significant heterogeneity, reflected by the differential expression of the lipopolysaccharide binding receptor (CD14) at their surface and the low-affinity receptor Fc, FcγRIII (CD16). In the last years, monocytes have been divided into three populations or subsets based on the intensity of CD14 and CD16 expression (cell surface marker phenotype) being functionally differentiated in: classical monocytes (CD14++/CD16−), present mainly in healthy patients; intermediate monocytes (CD14++/CD16+) and non-classical monocytes (CD14+/CD16++). A possible causal role in the development of atherosclerosis in general population and CKD patients has been attributed to intermediate monocytes (CD14++/CD16+) [59]. CD14+/CD16++ monocytes are inflammatory senescent cells characterized by their increased capacity to produce proinflammatory cytokines and because of their strong function as dendritic cells [60]. CD14+/CD16++ can be differentiated *in vitro* from CD14++/CD16− monocytes by a cellular senescence process. CD14+/CD16++ show senescent cells characteristics, such as an increased content of the enzyme β-gal or a shortened telomere length in comparison to monocytes CD14++/CD16−, and they accumulate in peripheral blood of elderly or CKD patients as a result of their resistance to apoptosis [7, 61]. Intermediate monocytes (CD14++/CD16+) are a developmental step between the classical monocytes (CD14++/CD16−) and non-classical (CD14+/CD16++) and whose activity is related to CVD [62, 63]. Moreover, non-classical CD14+/CD16++ monocytes appear to be involved in the endothelial damage which is usually by elderly people and CKD or others chronic inflamed patients [62, 63] leading to endothelial cells from the neighborhood achieve senescence status. Also, high frequency of CD14+/CD16++ ("non-classical") monocytes is associated with increased vascular superoxide production and apoptosis in endothelial cells [64, 65]. In normal states, the vascular endothelium does not allow the adhesion of leukocytes and prevents their passage. When hemodynamic conditions are altered monocytes, adopt a peripheral position along the endothelial surface producing adhesion of monocytes to the activated endothelium. The injury of endothelial cells is associated with the senescence of endothelial cell [66].

In vitro studies performed with CD14+/CD16++ in mature endothelial cells cultures, we found that those monocytes express high levels of vascular adhesion molecules, have a high adhesion capability to endothelial cells, produce chemokines, angiogenic factors and induce the production of vascular damage-associated MVs [7, 56]. MVs may contain molecules such as proteins, nucleic acids and lipids, which could contribute to the CVD development and also the profile of these molecules, are specific of the cell type of origin [67]. Thus, the accumulation of CD14+/CD16++ monocytes in peripheral blood not only can play a crucial role in the induction and can be responsible for prolonging the inflammatory response in elderly and CKD patients but can be directly related to CVD development. In CKD patients, we found that inflammatory monocytes are increased, mostly in those patients subjected to hemodialysis [68]. Proinflammatory or non-classical monocytes have a high binding affinity for endothelial cells conferred by their high expression of adhesion molecules. As a consequence, CD16-positive monocytes might preferentially adhere to the activated endothelium, enabling the propagation of further vascular damage by secretion of proinflammatory mediators [59].

In addition to the activity of the immune cells in endothelial damage, some other factors could be involved, as some specific molecules are known to be increased in the peripheral blood of CKD. In different models, it has been shown that endothelial cells activated pathologically with uremic serum or uremic toxins enter into a premature senescent state. Also, they reduce their proliferative capability and show shortened telomeres, augmenting the expression of β-gal [69]. Another possible factor in the development of the CKD-associated CVD is the incorrect repair of the damaged endothelium by EPCs. This failure occurs mainly due to two factors: a decreased number of EPCs or their imbalanced function. In our studies, we demonstrated that in CKD patients there is a decrease in the number of EPCs and that this number is considerably lower in severe patients with, for example, vascular calcifications [10, 70]. Also, it has been demonstrated that EPCs lose their angiogenic capability, generally needed in the process of regeneration of harmed vascular structures (vasculogenesis). In this regard, the association between some diseases such as CKD-associated CVD and both number and function of EPCs, accelerate the processes of EPCs senescence and therefore damage in endothelial cells harboring.

5. Microvesicles and endothelium

The endothelial MVs (EMVs) are extracellular vesicles produced by endothelial cells whose essential role is to act as a signaling system between the elements involved in the function and homeostasis of the vessel [71].

In general, the extracellular vesicles can be found in many body fluids, including plasma and urine. They have a variable size, between 0.05 and 5 μm [71], and are involved in physiological and pathophysiological processes, participating as mediators in intercellular communication. They can act directly on the target cells by binding to ligands, cell surface receptors and/or membrane-associated enzymes, delivering or releasing their contents directly into the cytoplasm. Extracellular vesicles are elevated in patients with neurodegenerative, metabolic, pulmonary, autoimmune and vascular diseases, chronic inflammation and cancer [72]. The use of extracellular vesicles as markers for the prediction, diagnosis and prognosis of the disease is increasingly interesting, as well as their potential as new therapeutic targets [73]. There are several types of extracellular vesicles: exosomes, the MVs or microparticles and the apoptotic bodies, which are produced by different mechanisms [65]. The MVs are a heterogeneous population of up 2 μm diameter, which are formed from the cell membrane in a regulated active process, dependent on enzyme activity and calcium.

Recently, it has been demonstrated that MVs may play an essential role in cellular senescence processes [74] since they have been proposed as elements of an endothelial response that can participate in the damaging and repair processes of the endothelium [10–12]. MVs generated from different cell types can induce endothelial dysfunction because they are responsible for increasing oxidative stress, reducing the bioavailability of NO and producing cardiovascular inflammation. The knowledge about their formation and release represent an attractive therapeutic goal to limit MVs levels, but the mechanisms underlying the release are not fully elucidated. On the other hand, a direct or indirect inhibition of the effect of MVs is a more effective proposal [75]. The effect of certain drugs that are used to decrease cardiovascular risk have been shown to affect the MVs plasma levels, suggesting that the beneficial effects of these

drugs could, at least in part, be mediated through a reduction of the concentration of MVs [76]. Moreover, different authors have highlighted the importance of diet on MVs release, being perhaps one of the mechanisms involved in the role of diet in the development of CVD [14, 77]. The process of identification and separation of extracellular vesicles is complicated due to their extensive variability. In fact, currently, the absolute separation of exosomes, apoptotic bodies and MVs is not possible because their size ranges may overlap. The most common method for the separation and isolation of extracellular vesicles is the serial centrifugation. In the majority of the studies, a first centrifugation is performed at 200–1500 × g to remove cells and cell debris. Extracellular vesicles more than 100 nm are pelleted at 10,000–20,000 × g and small vesicles of 100 nm at 100,000–200,000 × g [78]. Following these protocols, we can obtain EMVs from supernatants of mature endothelial cells cultures, cellular debris and exosomes-free. The EMVs might also be obtained from plasma by similar processes, but would be found mixed with other MVs derived from other circulating cells.

The most common methods to study single MVs are flow cytometry (FC), tunable resistive pulse sensing (TRPS), dynamic light scattering (DLS) and nanoparticle tracking analysis (NTA) [79]. To date, FC is the method most used to establish the cellular origin and the phenotype of the MVs and is based on the detection of light scatter and fluorescence intensity of the labeled MVs [80–82]. To characterize their cellular origin, different antigens expressed on the membrane of the MVs are identified. For this purpose, monoclonal antibodies (mAb) labeled with different fluorochromes that define the phenotype are used. To identify EMVs, specific fluorescent antibodies against endothelial cell can be used to characterize the phenotype. Some markers used to describe EMVs are CD144, CD105 and CD146. Moreover, the phospholipids are a class of lipids that are a major component of all biological membranes and in MVs are externalized. For this reason, these phospholipids present in the MVs membrane have also been used for EMVs detection and characterization [83]. The combination of several mAb simultaneously can facilitate the identification of the origin and the state of activation or apoptosis of the cell from which the MVs originate [84]. The EMVs determination protocol includes some preliminary steps designed to identify sizes, with beads that allow adjustments to the equipment, before the introduction of the samples. However, this method has limitations in identifying the smallest MVs that are below the detection limit of conventional FC equipment (diameter size lower 300 nm) [79]. Recent studies have shown that FC equipment with high sensitivity can amplify the forward scatter parameter capacity, which is used to identify the size of the MVs [85]. On the other hand, it is very helpful to provide information regarding functional activity of the extracellular vesicles [86–89].

In this regard, novel instruments including NTA or DLS have shown their advantages in the analysis of extracellular vesicles. NTA measures the distribution of the absolute size of the vesicles that range from 50 nm to 1 μm [90]. The vesicles in suspension are illuminated by a laser that produces light scattering or fluorescence. A microscope determines the position of individual vesicles, which are continuously moving due to Brownian motion [91]. When a fluorescent marker is used, NTA can also be used to determine the size of a subgroup of vesicles [92]. The principal advantage of this method is the detection of particles below 100 nm in diameter. In contrast, the limitation of this technique, the low resolution, therefore, NTA is incapable of distinguishing MVs from particles in suspension (debris) with the same size [79]. DLS, also known as photon correlation spectroscopy, measures the size distribution of vesicles between 1 nm and 6 μm. However, the absolute concentration of the vesicles cannot be determined by DLS because the average amplitude of the signal depends on the diameter, concentration and the refractive index of the vesicles [93–95].

The last method TRPS consists in the movement of the MVs through tunable nanopores which are capable of registering MVs between 80 and 1000 nm [96]. Particles passing the pore generate a change in the electric resistance, thus providing information on diameter, surface charge and concentration of single particles. The major disadvantage of TRPS is that it cannot distinguish between MVs and similarly sized particles [79]. Independently of the method used to study of the MVs, it has been recommended to confirm the presence of MVs by measuring them at least with two different techniques.

In addition, enzyme-linked immunosorbent assay (ELISA), Western blot or quantitative real-time PCR (qPCR) are useful tools for the detection of proteins or RNA in preparations of purified MVs. Electron microscopy can provide information concerning the vesicular morphology, size and the presence of markers. Moreover, proteomic analysis and profiles of RNA/microRNA (miRNA) may help to determine the composition of the MVs.

In the absence of pathology, the EMVs are involved in the maintenance of vascular homeostasis, participating in the metabolism of the vascular environment [97]. The EMVs can act on the vascular wall, at the endothelial level, and on smooth muscle cells [98], regulating both vasomotor reactivity and angiogenesis. In fact, the formation of EMVs and their elimination seems to reflect a balance between activation and cell damage, cell survival/apoptosis and angiogenesis. Endothelial responses may be immediate; releasing various factors or can be delayed, modulating the expression of genes involved in regulating the structure and function of the vascular system (**Figure 2**). In *in vitro* models, endothelial cell cultures produce EMVs in a meager percentage without additional stimulus. However, in response to activation

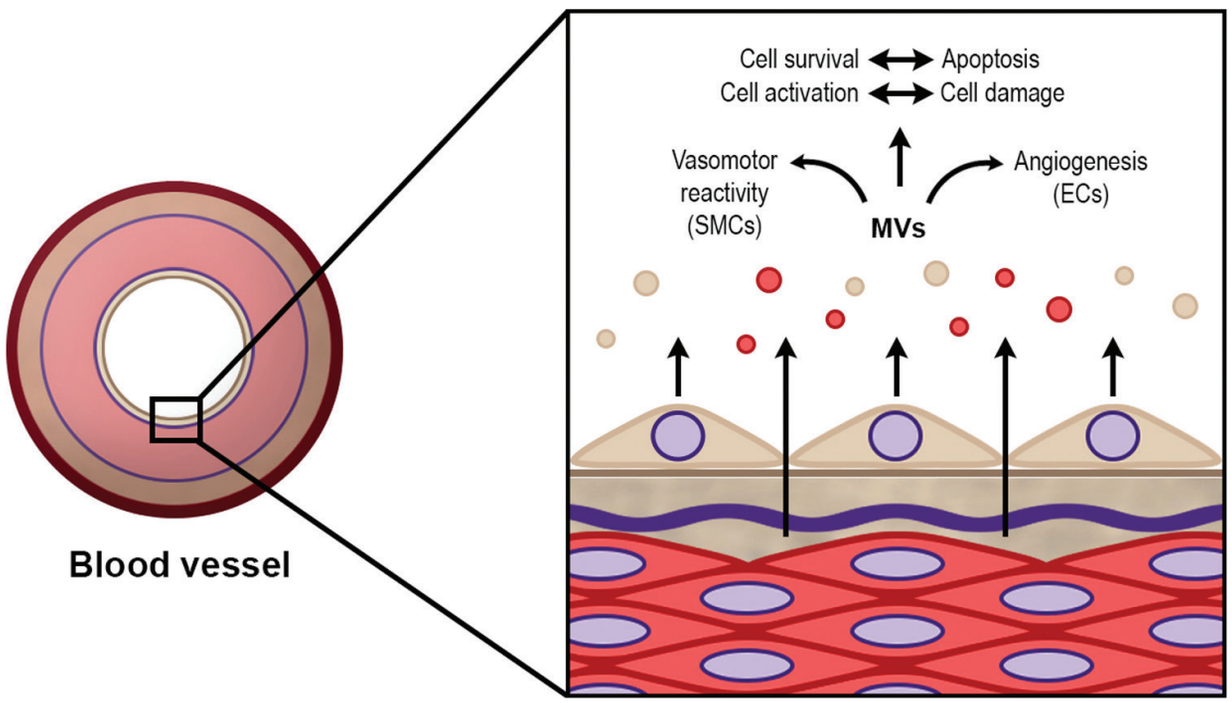

Figure 2. Mechanisms of endothelial microvesicles (MVs) action upon target cells. SMCs, smooth muscle cells; ECs, endothelial cells.

processes and/or apoptosis, the number of EMVs increases significantly. Physiological blood levels of EMVs present in healthy individuals are between 10^3 and 10^4 EMVs/mL and pathological concentrations (present in individuals with CVD) are 10^5 EMVs/mL [99]. Several authors have found that mature endothelial cells in culture, exposed to activation by cytokines, released more EMVs [100, 101].

MVs concentration in blood from healthy subjects is clinically irrelevant. However, in patients with cardiovascular risk factors and after cardiovascular events, EMVs concentrations are increased significantly [10, 102]. In fact, in patients with CVD, an association between the number of circulating EMVs and the Framingham risk score has been shown [72]. In particular, high levels of EMVs in diseases associated with vascular injury seem to reflect an inflammatory and prothrombotic process. EMVs may participate in the development and amplification of CVD through both cardiac and vascular cells. On the other hand, numerous studies have emphasized the effect of cardioprotective drugs on reducing concentrations of extracellular vesicles [73] which reinforces the evidence about the possible correlation of EMVs and vascular injury.

EMVs, and in general all extracellular vesicles, carry a specific load that is capable of delivering to other cells, even in remote locations. Extracellular vesicles share characteristics with their parental cells such as cell surface receptors, integral membrane proteins, cytosolic molecules, organelles, mRNAs, miRNAs or small amounts of DNA and proteins, including transcription factors, cytokines and growth factors [103]. Cell receptors and transmembrane proteins can help in the identification of EMVs, and also are indicative of the ability of vesicles to interact directly with receptors on the surface of target cells, resulting in an intracellular signal transmission. In addition to its effect on specific receptors, it has been shown that EMVs may be fused to the target cell and transfer its contents directly inside as a vehicle for transfer of genetic information [11, 67, 104, 105]. Extracellular vesicles are considered as the main source of miRNAs, released into the bloodstream during cell activation or apoptosis [106]. In fact, most miRNAs are associated with extracellular vesicles and only small amounts of them can be found free in plasma. It is thought that extracellular vesicles are necessary to protect circulating miRNAs from degradation by RNases, transferring safely functional miRNAs from the parental cells receptor cells. miRNAs act as regulatory molecules in endothelial cells, vascular smooth muscle cells, platelets and inflammatory cells that contribute to modulate the initiation and progression of atherosclerosis. It is known that the release of miRNAs does not occur randomly but they are produced and released by controlled mechanisms [107, 108]. It has been described that there are several miRNAs involved in the regulation of vascular function and repair. It is expected that in the future, a better understanding of these molecules provides new options both diagnostic and therapeutic in the vascular pathology.

The MVs from different sources such as endothelial cells, monocytes and lymphocytes can promote oxidative stress in the endothelium through processes that may involve several enzymatic systems [109]. The MVs can regulate the production of reactive oxygen species (ROS), although there are some discrepancies regarding ROS generation systems affected. These contradictory results may be due to the fact that MVs populations studied are from different sources or produced by different stimuli [105, 110]. From the biological point of

view, these differences in the production of MVs have a significant for the potential to define MVs populations with different biological activities.

One of the best-provided properties of MVs is its ability to promote coagulation [98]. In fact, the MVs are elevated in hypercoagulative disorders probably as a result of their active participation [98]. It is not clear how far MVs contribute to the *in vivo* coagulation, but there are several *in vitro* studies that demonstrate their procoagulant role. This capacity has been extensively studied in platelet-derived MVs, but the fact is that the MVs have two specific and common physical characteristics that may be responsible for this procoagulant activity: firstly, the externalization of phosphatidylserine as coagulation promoter and secondly, the expression of tissue factor, which is a critical component of the early stages of coagulation [11]. Indeed, tissue factor is not expressed under physiological conditions in circulating and endothelial cells, but it is expressed in pathological conditions.

Chronic inflammation is a crucial factor in the development of atherosclerosis, and the effects of EMVs in inflammatory processes have been the subject of numerous studies since they may represent both a cause and a consequence of inflammation [12]. The MVs isolated from human atherosclerotic plaques can transfer intercellular adhesion molecule-1 (ICAM-1) to endothelial cells and could increase the ability to recruit inflammatory cells in a manner dependent of phosphatidylserine, which may increase the progression of the atherosclerotic plaque. The most conclusive evidence of a proinflammatory role for EMVs is that the administration of exogenous EMVs to rats is associated with acute lung injury, with increased levels of proinflammatory cytokines (IL-1β and TNF-α) and neutrophil infiltration on histological lesion perivascular space [111].

Different studies have described a role of MVs in the regulation of angiogenesis [112]. Platelet-derived MVs were first involved in the angiogenesis process since platelets contain at least 20 factors that regulate angiogenesis. Platelet-derived MVs stimulate proliferation, survival, migration, and formation of capillary-like structures in endothelial cells *in vitro*. Furthermore, injection of platelet-derived MVs increases myocardial post-ischemic capillary density in rats [113]. Subsequent studies have shown that MVs isolated from atherosclerotic plaques are involved in the formation of new blood vessels and in the progression of the plaques to rupture. Endothelial cells in the culture containing MVs that release matrix metalloproteinases (MMP-2 and MMP-9) and promote matrix degradation and the formation of new blood vessels.

In addition to being a potent stimulus for the formation of MVs, apoptosis can also be a consequence of MVs signaling [112]. Monocyte, erythrocytes, platelets and endothelial cells-derived MVs contain caspase-3. It is thought that the content of caspases may be a mechanism directed to control the apoptosis, suggesting that MVs could release caspase-3 into the target cells, participating in the induction of apoptosis. In addition, caspase-3 is implicated in numerous cellular processes, so the release of this protein could have an even more significant impact on the target cell.

The MVs contain proteolytic enzymes, and then some of its effects could be attributed to alterations in the extracellular matrix or proteolytic cleavage of various signaling molecules. For example, the microvasculature-derived EMVs containing MMP-1, MMP-2, MMP-13 and MMP-7, which degrade fibronectin *in vitro* [114]. Moreover, MVs isolated from human atherosclerotic plaques contain an active form of ADAM17 (metallopeptidase domain 17), an enzyme with a role in the control of inflammation and tissue regeneration. This enzyme could

contribute to the release of cytokines and the development of alterations mediated by MVs in the extracellular environment [115].

Initially, proliferation and migration of adjacent endothelial cells have been identified as a factor of endothelial repair, and subsequent studies have shown that the maintenance of the endothelial structure is associated with EPCs and their ability to differentiate and repair damaged endothelial tissue. Due to the importance of this repair mechanism in the maintenance of vascular homeostasis, it is logical to think about the existence of close communication between damaged endothelial cells and EPCs. Previous studies performed by our group suggest that plasma EMVs, both of healthy subjects and patients with CKD; participate in the activity of the EPCs [10]. Our hypothesis is that EMVs can be an essential and necessary physiological mechanism of signaling to initiate the recruitment of EPCs from bone marrow. In *in vitro* models, we have shown that EMVs may be the key element in the regeneration and maintenance of vascular homeostasis, acting on EPCs [116]. Indeed, in response to different stimuli, the endothelial cells can induce EMV with different membrane characteristics, miRNA and other molecules in your content that reduce the ability of EPC to regenerate and participate in the signaling pathways involved in apoptosis and oxidative stress [117]. These specific mechanisms may constitute therapeutic objectives in future studies.

Vascular calcification is an increasingly constant process in developed countries and can contribute significantly to increased cardiovascular risk. The processes and mechanisms involved in the formation of vascular calcifications are poorly understood and are needed to develop new therapeutic strategies to prevent or avoid calcification. Patients CKD have a higher incidence of vascular calcification, and our group has shown that EMVs are increased in patients with an elevated degree of calcification [10]. In *in vitro* studies, EMVs produced in an inflammatory environment or obtained from patients with CKD promoted the calcification of smooth muscle cells, as assessed by some calcification markers (bone morphogenetic protein-2 (BMP-2) and alkaline phosphatase (ALP)) and the phenolsulfonephthalein method [100]. Other authors have also described a role of the MVs in the mineralization of vascular smooth muscle cells [118].

MVs have also been associated with endothelial senescence. As we said before, senescent cells release characteristic molecules and substances composing the SASP. However, some of those substances which are known to be part of this SASP cannot be released as soluble molecules due to their nature, as some transmembrane proteins [119]. It is known that the premature induction of cellular senescence *in vitro* increases the release of extracellular vesicles [120]. Those concepts suggest the contribution of MVs as part of the SASP, which have two important consequences: (1) SASP MVs can be the mechanism by which those insoluble proteins are released and (2) the carrier molecules can activate signaling processes in the target cells. Nevertheless, the specific mechanisms underlying MVs releasing from the senescent cells are still unresolved. It has been described that p53, a tumor-suppressor protein, remarkably upregulated in senescence, modulates the release of extracellular vesicles [121]. Also, p53 takes part in the transcription of some molecules implicated in extracellular vesicles biogenesis, partly explaining how senescence and MVs releasing activation can be related [122–124]. Moreover, the content within those MVs may be necessary in the induction of senescence in the target cells, as it has been shown that some miRNAs can regulate the p53 and pRB pathways [125–127]. Loss of pRB results in deregulated cell proliferation and apoptosis, whereas loss of p53 desensitizes cells to checkpoint signals, including apoptosis [128]. Thus, the presence of those miRNAs in MVs may be associated with

hormonal changes driving aging (endocrine senescence induction) playing a critical role in the aging process and adding a new perspective on the mechanisms involved in aging.

6. Conclusions and perspectives

CVD seem to begin as a consequence of a damaging process and endothelial dysfunction, and there are pieces of evidence implying cellular senescence in the functional imbalance of the endothelium. Cellular senescence is a physiological mechanism which occurs as a consequence of aging, but

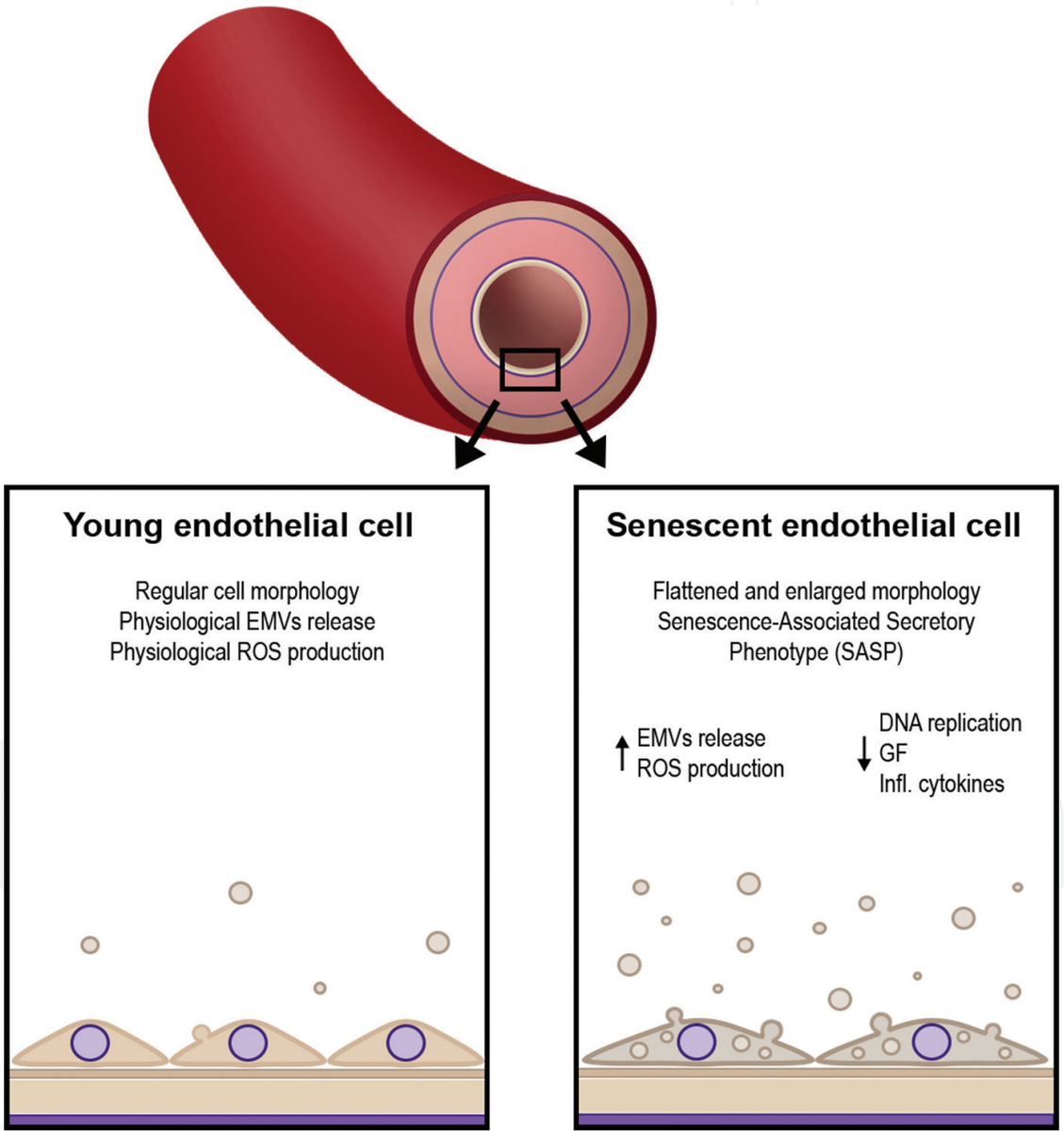

Figure 3. Different characteristics of young and senescent endothelial cells. Senescent cells undergo distinctive phenotypic, morphological alterations and senescence-associated secretory phenotype (SASP). The number of endothelial microvesicles (EMVs) of the senescent cells is greater than those derived from young cells. Also, the reactive oxygen species (ROS) production is higher in senescent endothelial cells compared with young endothelial cells. Moreover, the secretion of growth factors (GF) and proinflammatory cytokines (infl. cytokines) from senescent endothelial cells are reduced.

under different pathologic conditions, its regulation is modified, as in CVD or CKD. Senescent endothelial cells change their morphological and functional characteristics (**Figure 3**) and cannot correctly regulate the repairing and regenerative activity of EPCs. In the endothelial senescence context, the role of EMVs appears to be important. EMVs are considered as biomarkers of endothelial injury and are associated with an inflammatory and prothrombotic state. However, the perspectives of their study are beyond their role as biomarkers, as they are capable of transmitting biologic information in several physiologic and physiopathologic processes. EMVs are increased in elderly, but also in patients with CVD and CKD. Many questions remain unresolved to understand the role of EMVs in the endothelial function and damage. To comprehend and characterize the mechanisms by which the senescent endothelial cells show an imbalanced functionality is of great interest, opening new perspectives to increase our knowledge and to identify useful biomarkers in the timely diagnostics and to design therapeutic objectives in CVD.

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