REVIEW ARTICLE

Methods of Endothelial Function Assessment: Description and Applications

Amanda Sampaio Storch, João Dario de Mattos, Renata Alves, Iuri dos Santos Galdino, Helena Naly Miguens Rocha

Universidade Federal Fluminense, Niterói, Rio de Janeiro, RJ – Brazil

Introduction

The endothelium is a monolayer of cells, called endothelial cells, that lines the interior of blood vessels, including arteries, veins and cardiac chambers,[1] acting as a protective layer between circulating blood and other tissues.[2] The endothelium is crucial for the control of vascular homeostasis, and is involved in the regulation of intracellular signaling,[1] vascular tonus and permeability,[3] coagulation cascade and angiogenesis,[4] among others. One of the main activities of the endothelium is the release of autocrine and paracrine substances in response to stimuli.[2] Injuries to the endothelium trigger an inflammatory response with participation of several cell types – lymphocytes, monocytes, platelets and smooth muscle cells[5] – culminating in endothelial cell dysfunction, stiffness of vessel wall and atherosclerotic plaque formation.[6]

Endothelial dysfunction is an early, key characteristic of development and progression of atherosclerotic plaque and subsequent complications. This is characterized by a reduced bioavailability of endothelium-derived vasodilators, such as nitric oxide (NO), along with a relative or absolute increase in available vasoconstrictors. Such unbalanced condition impairs the endothelium-dependent vasodilation, a functional marker of endothelial dysfunction.[7]

At the beginning of atherosclerotic plaque formation, endothelial dysfunction is characterized by increased expression and release of adhesion molecules, including endothelial selectin (E-selectin), intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1). These molecules are released in response to stimuli of inflammatory cytokines, bacterial lipopolysaccharides and oxidized low-density lipoproteins (ox-LDL). They promote cell-cell and cell-extracellular matrix adhesion, leading to foam cell accumulation on the subendothelial space,[8] increased vessel wall thickness and consequent reduction or even complete obstruction of vascular lumen.[9]

Assessment of endothelial function consists of the analysis of endothelial cells responsiveness to vasodilator or vasoconstrictor stimuli, contributing to advances in the understanding of atherosclerosis and possible therapeutic targets.[2] The methods include *in vitro* analysis, such as culture of endothelial cells, and *in vivo* analysis, such as flow-mediated dilation (FMD), venous occlusion plethysmography (VOP) and measurement of serum markers. However, none of these methods have been currently applied in the clinical setting, due to invasiveness, high costs and difficult standardization of the techniques.[1]

Endothelial dysfunction precedes morphological atherosclerotic changes and may contribute to the development of lesions and clinical complications. Cardiovascular diseases (CVD) are the main cause of natural death in the world, including in Brazil. Recent data shows CVD accounted for approximately 30% of deaths in the country. In this context, the use of the aforementioned techniques in clinical research allows a better understanding of this problem and the development of new prevention and treatment strategies to decrease morbidity, mortality and also public costs. A deep understanding of endothelial dysfunction assessment tools by healthcare professionals allows the improvement of these techniques and enables the transition from clinical research to clinical practice.

Here we describe the most recent methods for the assessment of endothelial dysfunction.

Keywords

Endothelium, Vascular; Function, Endothelim; Atherosclerosis; Vasodilatation.

Mailing Address: Amanda Sampaio Storch
Universidade Federal Fluminense - UFF
Rua Professor Hernani Pires de Melo, 101 São Domingos, Niterói, RJ – Brazil
E-mail: amandasstorch@gmail.com

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In vitro analysis

Endothelial cell cultures

Culture of endothelial cells has been widely described in the literature, especially in studies on the effect of oxidative stress and inflammation on mobilization and proliferation of these cells *in vitro*.[1] The yield of endothelial cells culture from peripheral blood represents an important step in research on circulating angiogenic cells, since these cells were firstly isolated for research purpose.

However, the use of cell culture for assessment of endothelial function has its pros and cons. The possibility of investigating a wide variety of intracellular signaling pathways allows the development of different studies, with different purposes. For example, the discovery of surface markers, monoclonal antibodies, and magnetic beads for immunoassays has facilitated the isolation, quantification and characterization of endothelial cells. However, this process has limitations related to phenotypic changes of the cells, dependent on cell cuture media and time. Also, the culture of endothelial cells has not been fully established as a model comparable to *in vivo* ones.[10] Therefore, the protocol for cell culture should be carefully prepared, since many growing factors have been recognized to stimulate different phenotypes in proliferating cells.[11]

Due to the difficulty in studying the endothelium *in vivo*, several *in vitro* techniques have been developed.[12] Among these, human umbilical vein endothelial cells (HUVECs) have been widely used as a source of human endothelial cells, since they are free from pathogens and are physiologically more relevant than other available lineages.[13] Primary HUVECs preserve endothelial cell native characteristics, including the expression of specific surface markers and intracellular signaling pathways.[14]

In culture media, HUVECs change from a dispersed to a clustered pattern within days of incubation, covering most of the bottom surface of the plate.[15] Once the plate is completed covered, the cells polarize, form tight junctions, and finally achieve the cobblestone stage, which mimic the in vivo condition.[16] HUVECs have been used in molecular biology research, contributing to advances in the pathophysiology of atheroma plaque formation and mechanisms involved in the control of angiogenesis and vascularization of affected areas.[12,17] Cultures of HUVECs are applied in studies on cell interactions (resulting in analysis of adhesion molecules and cytokines), effects of shear rate and oscillatory flow in cell signaling (mimicking what occurs in the vessel lumen), and allows the discovery of receptors and transcription factors involved in the development and progression of endothelial dysfunction.[18-20] However, caution is needed in interpreting the results because of the limitations of this method.

In vitro functional studies

In the last decades, different techniques have been used to assess endothelial function *in vitro* including the organ bath technique, and myographic recordings, and shown to be essential for the discovery of endothelium derived relaxing factors (EDRFs). The most widely studied EDRF is nitric oxide (NO).[21] These studies allow the measurement of endothelium-dependent relaxation of vascular in response to well-known agonists, such as acetylcholine (ACh) and sodium nitroprusside.[22]

Isolated organ bath is an *in vitro* technique that assesses vascular reactivity in response to agonists, yielding a dose-response curve used to investigate physiological and pharmacological responses of biological preparations isolated from a variety of species (rabbit, rat, etc). Tissues and organs used in this method include artery and vein rings or strips, atrium, ventricle, papillary muscle, diaphragm, fundus of stomach, small bowel (duodenum, jejunum, and ileum), trachea, uterus, among others. The method aims to mimic an optimal physiological condition regarding temperature, aeration and nutrients for analysis of these preparations. An organ bath system is composed of a double-jacketed glass chambers with capacity varying from 5 to 50mL connected to a helical warming coil through which the nutritive solution is conducted. The warming coil was submerged in water maintained at 37°C, which was perfused with a peristaltic pump. The isolated organ or tissue is maintained in muscle chamber; one end was attached to a glass rod connected to an air pump that provided aeration of the nutritive solution, and the other end was fixed to a lever connected to a force transducer by a cotton thread.[23,24]

From this point on, the experiments have involved the cumulative addition of drugs to the muscle chamber or electrical stimulation (when the organ was mounted between to platinum electrodes connected

to an electrical stimulator), resulting in contraction or relaxation of the muscle studied. Changes in tension are recorded by myographs (kymograph, physiography or digital system),[1] that register the intensity and kinetics of different contraction stages (velocity, frequency or decay). Dose-response or stimulus-response curves are generated from the experimental results.[23] Displacement of the dose-response curve shifts to the right indicates impairment of EDRF/NO release in the endothelium.[25]

The organ bath technique is hence an important method for enabling the control of experimental conditions – nutritive solution, temperature, etc. – and isolation of endothelial function elements by using, for example, endothelial nitric oxide synthase (eNOS) inhibitors (e.g. L-NAME). Other advantages of the method include a relatively simple preparation, which allows the analysis of simultaneous preparations; the possible use of electrical stimulation; and an accurate quantification of the response.[23]

One disadvantage of this method is the fact that its use is restricted to animal experimental studies, due to ethical considerations regarding the required availability and amount of human samples, and to the variability of patients' responses to controlled events. Another one is the necessity to use relatively large tissue samples (>1 mm diameter), which limits the assessment of smaller diameter vessels.[23] Although this has been the most widely used method for endothelial function assessment in animal models, one major limitation is to not distinguish the biological processes occurring in the vessel lumen and outside the blood vessel.

In vivo analysis

Flow-mediated dilation (FMD)

There is strong evidence supporting that the detection of brachial artery endothelial dysfunction is an important indicator of systemic endothelial dysfunction.[26] Vascular function can be measured by several methods, including invasive and non-invasive techniques.[27]

Invasive techniques consist mainly of intra-arterial administration of compounds that stimulate NO release, which induces dilation of vascular beds in healthy subjects. In this situation, detection of vasoconstriction may indicate endothelial dysfunction.[28] Nevertheless, the invasive nature of these techniques unables their use in the general population and, for this reason, non-invasive methods could be more practical and more accessible alternatives for measurement of vascular function.[27]

Among the non-invasive techniques for assessment of vascular function, the most important, and most commonly used combines arterial imaging (especially of brachial artery) in response to reactive hyperemia, causing a flow increase and expected endothelium-dependent dilation.[22,27] In this method, an arterial occlusion cuff is inflated for occlusion of arteries in the distal hand or distal forearm, followed by deflation allowing vasodilation in response to reactive hyperemia in distal and proximal vascular beds, which is mediated by release of endothelial factors, such as NO.[29] NO induces increased shear stress and dilation, resulting in increased flow in the proximal artery.[22] Such response is known as flow-mediated dilation (FMD). The method involves ultrasound arterial imaging in two conditions, at rest (baseline) and during reactive hyperemia (after a five-minute arterial occlusion).[27,30]

Brachial FMD is an indirect measure of NO release by the endothelium due to a transient flow stimulus.[31] Dependence of FMD on NO was demonstrated by intra-arterial infusion of NG-monomethyl-L-arginine (L-NMMA), a specific eNOS inhibitor, which decreased arterial dilation by approximately 66% in response to shear stress.[32]

A proportional relationship between the magnitude of dilation and endothelial function has been suggested.[22] Impaired FMD is one of the early, reversible manifestations of vascular disease, and may be an important indicator of endothelial damage.[33] In addition, endothelial dysfunction detected by FMD can potentially predict a risk factor even in the absence of coronary diseases.[26,34] Assessment of FMD has been proposed as a method with potential clinical applicability in the identification of risk factors for cardiovascular events, even in asymptomatic patients.[35]

Furthermore, measurement of FMD can stratify individuals at low, moderate or high risk for future cardiometabolic events.[31] Gokce et al.[36] have suggested that impaired FMD has long-term prognostic value and that the technique may be used to influence therapeutical strategies on a particular patient. In this context, studies have demonstrated an association between impaired FMD and risk factors for cardiovascular events.[34-36]

In methodological perspectives, some aspects should be taken into account in assessing FMD. Due to variations in

dilation between post-prandial periods, particularly after high fat meals and fasting periods, or during menstrual cycle, it is recommended that FMD be assessed during fasting and at the same menstrual cycle phase (folicular phase).[33]

However, similar to other methods, FMD has a few limitations that should be considered. First, the lack of standardization and variations in cuff placement or positioning make comparison of results difficult. Besides, in some situations, the degree of reactive hyperemia may vary even under the same stimulus. Also, changes in the structure of blood vessels and impaired dilation may be limiting factors during FMD assessment.[1] It is worth pointing out that the method is highly observer-dependent, which may lead to different FMD values.

Laser Doppler flowmetry (LDF)

LDF is an excellent method for measuring skin microcirculation.[37,38] The method is based on the direction of a laser light beam to a tissue sample at a known frequency and measurement of the Doppler shift that arises in light that has been scattered by moving red blood cells.[22] LDF has been widely used because of its non-invasiveness, simplicity and possibility of continuous measurement.[37] LDF does not provide a direct measurement of blood flow, but rather an index of cutaneous perfusion.

The technique is based on diffusion and refraction of a monochromatic light beam. The light has its wavelength changed (Doppler effect) after being scattered by moving red blood cells.[38] The frequency and size of this effect are associated with velocity and amount of red blood cells. The wavelength commonly used in laser-Doppler flowmeter is 780 nm, which can penetrate into the skin regardless of color or oxygen saturation.[38,39]

When combined to other techniques, such as the infusion of vasoactive substances, iontophoresis of small charged molecules and techniques that induce reactive hyperemia, LDF has been used to determine changes in skin microcirculation at real time.[22] For example, LDF combined with the intradermal administration of ACh by iontophoresis induces endothelium-dependent dilation that allows quantification of local blood flow.[40]

LDF has good precision in quantifying rapid changes in cutaneous blood flow. However, the characteristic heterogeneity of the tissue, due to differences in anatomy, causes spatial variability that leads to a relatively low reproducibility of the method. On the other hand, the use of integrated probes may reduce spatial variability and increases in reproducibility.[38] Another advantage of this technique is its non-invasiveness; even when combined with intradermal infusion, the method is relatively safe due to the small amount of drug infused. LDF is less challenging than FMD, but has good clinical potential for assessment of vascular health.[22]

Venous occlusion plethysmography (VOP)

VOP has been studied for more than 90 years, and its primary objective was to evaluate blood flow in the organs.[41] Today, VOP is widely used in the evaluation of endothelial function, in the assessment of the autonomic nervous system that regulates blood flow, and the vasodilation response to several stimuli, such as exercise and stress.[41,42] For its easy applicability, VOP has been widely used in *in vivo* studies on vascular physiology in humans, both in healthy individuals and in several disease conditions.[43]

VOP is a non-invasive method that involves inflation of the upper arm cuff to a pressure lower than systolic pressure. Usually, an inflation pressure of around 40 mmHg is used for intervals of approximately 10 seconds, followed by 5 seconds of deflation, during 2-3 minutes[44,45]

The inflation pressure used is sufficient to arrest venous return without impairing arterial flow towards the forearm, causing a linear increase in blood flow to the limb. This is measured by a strain gauge, which detects any change in tissue volume, placed in the point of maximal circumference of the forearm.[43]

At resting conditions, blood flow in the forearm is divided into 70% in striated skeletal muscle and 30% in the skin. There are multiple arteriovenous communications in the hand related to skin supply. To avoid that such blood supply affects the measurement of forearm blood flow, a wrist cuff is inflated to suprasystolic pressure just before flow measurement.[42,43,46,47]

Vessel responsiveness is assessed by evaluation of volume changes in the limb in response to stimuli, which can be a mechanical stimulus that promotes reactive hyperemia, or a chemical stimulus via administration of vasoactive compounds (invasive technique). A decreased or impaired response may be an evidence of endothelial dysfunction,[48] which is considered a critical factor for the development, progression and complications of coronary artery disease.[49] Preserved endothelial function acts as an important regulator of vascular inflammation and remodeling.[43,47,48,50]

Endothelium-dependent vasodilation can be measured by reactive hyperemia which is induced by inflation of a forearm cuff as described above. The stimulus triggers the release of vasodilators, particularly NO, in the microvasculature, and increases flow velocity. The shear stress resulting from the friction of blood against the wall of the vessel promotes vasodilation. The cuff is rapidly deflated and changes in the vessels are measured.[50] This response can also be measured by administration of ACh, which is an agonist of muscarinic receptors located on the endothelium, and used in the assessment of endothelium-dependent vasodilation. Usually, increasing doses of ACh are administered during 5 minutes and the forearm blood flow is measured (as $mL.100mL^{-1}.min^{-1}$) in the last two minutes of infusion.[43,47]

Endothelium-independent vasodilation is evaluated by the infusion of vasoactive substances that act as NO precursors, such as sodium nitroprusside, for example. Vascular changes will be detected by strain gauges, which confirm whether an adequate response has occurred. When the response is consistent with the stimulus, the endothelial function is preserved.[43]

Invasive VOP, i.e., involving intra-arterial infusion of vasoactive substances, is considered a gold standard tool for analysis of vascular behavior, but is largely impractical in the clinical setting.

Pulse wave velocity (PWV)

At the end of ventricular ejection, a pressure wave propagates from the heart to the periphery at certain velocity. This velocity is named PWV.[51] Some authors have defined PWV as the distance traveled by blood flow divided by the time taken to travel that distance (in meters/second).[52]

PWV has been used as an important marker of cardiovascular risk.[53] The lower the PW, the more elastic and healthier the vessel. However, increased arterial stiffness is related to increased propagation of pulse waves in the aorta and large vessels, and early return of pulse waves from the periphery.[54] Pulse waves are normally reflected at discontinuities throughout the arterial tree, and retrograde waves are generated in the ascending aorta. This early return in the end of systole causes a cardiac overload.[51]

The association between arterial stiffness and risk factors such as coronary artery disease, diabetes mellitus, arterial hypertension, diastolic function and age has been well established.[51] PWV values are lower in healthy young individuals, but increases with age, as the elastic properties of blood vessels decrease.[55] The cutoff value of PWV for prediction of cardiovascular events is still controversial, but a value greater than 12 m/s has been suggested as indicator of cardiovascular risk.[51]

Technical differences exist between the methods for PWV measurement. However, the carotid-femoral relation has been considered the gold standard in the literature, for its non-invasiveness and for expressing PWV values directly related to the aorta artery.[56-58] The method is performed with the individual in the supine position, and two transducers are used for the measurement, one is placed on the right common carotid artery and one on the right femoral artery. The difference between the time for the onset of carotid pulse wave and femoral pulse wave, and the distance between the two transducers are used to calculate PWV.[51,57,58]

Another method that has been widely recommended for pulse wave analysis is applanation tonometry. This technique is used to estimate aortic pulse wave from the common carotid artery or the radial artery pulse waves. However, as radial artery is supported by a bony structure, which makes the measurement easier, radial artery tonometry has been the most commonly recommended approach. A transducer is placed on the skin at protruding parts of the vessel to record pressure wave signals, and then a transfer function is applied to calculate aortic pressure wave.[57]

Since arterial stiffness is a strong evidence of cardiovascular disease, analysis of pulse wave in the clinical practice may identify changes that might be related to vascular health, even before the occurrence of signs and symptoms.[58]

Limitations of the method are related to associated comorbidities, including metabolic syndrome, obesity and diabetes, which may affect the femoral pulse wave recordings. Besides, men with abdominal obesity and women with large breast size can cause errors in distance measurements.[57]

Serum markers of endothelial dysfunction

Biomarkers are analytic tools used in the analysis of biological parameters. In 2001, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker or biological markers as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic

processes, or pharmacologic responses to a therapeutic intervention". Today, biomarkers have been used in: 1) identification of individuals at high cardiovascular risk; 2) fast and accurate diagnosis of diseases; 3) effective prognosis and treatment. Regardless of its purpose, a biomarker has clinical relevance when it is accurate, standardized and reproducible, appropriate for the patients' condition, of easy interpretation by clinicians, and if it has high sensitivity and/or specificity for the parameter it proposes to identify.[59]

Advantages of the use of serum biomarkers in the assessment of endothelial function include simplicity of the procedures and the fact that samples of venous blood are widely used in laboratory routine. The use of these biological markers in the prognosis and/or diagnosis of vascular diseases is most common at initial stages, but this is an area of great potential.[1]

Cell adhesion molecules (ICAM-1, VCAM-1 and E-selectin)

Endothelial cells play a key role in the transport of another cell types and metabolic substrates between blood and interstitial space, including a complex signaling system that regulates innate immune responses in the vascular bed. When these cells are activated by pro-inflammatory stimuli, such as bacterial endotoxins, interleukin 1B (IL-1B), tumoral necrosis factor alpha (TNF-α), C-reactive protein (CRP), and ox-LDL, or by blood flow-related hemodynamic forces, the expression of adhesion molecules is increased and, thus, are considered early markers of endothelial activation and systemic inflammation.[60]

Transendothelial migration of leukocytes is regulated by soluble cell adhesion molecules, such as ICAM-1, VCAM-1 and E-selectins. ICAM-1 is a member of the immunoglobulin supergene family and ligand for the β2 integrin molecules present on leukocytes,[61] and is highly expressed in endothelial cells and subendothelial macrophages.[62] ICAM-1 mediates a number of cell-cell interactions, including adhesion and transmigration of leukocytes to the vascular endothelial wall. Data from the literature support the hypothesis that ICAM-1 expression activates endothelial cells and leads to inflammation, which, in turn, are important steps for the initiation and progression of atherosclerosis.[63]

VCAM-1 also belongs to the immunoglobulin supergene family and is ligand to the very late antigen-4, a β-integrin found on the surface of mononuclear cells.[64] VCAM-1 expression is restricted to endothelial cells and occasional fusiform cells. In contrast to ICAM-1, which is produced in low concentrations, VCAM-1 is not expressed on healthy endothelial cells.[62] It has been suggested that VCAM-1 expression may result in endothelial activation, as it increases the recruitment of monocytes and improves the monocyte-endothelial interaction in the initial steps of the atherosclerotic lesion formation.[65]

E-selectin, a molecule belonging to the family of C-type lectins, is probably the most specific endothelial activation marker.[66,67] Its expression is restricted to vascular endothelium and induced by inflammatory cytokines. This adhesion molecule is involved in leukocyte recruitment to inflammation sites, and participates in slow leukocyte rolling in inflamed venules.[67]

Inflammatory markers

Recent evidence has demonstrated that inflammation is closely related to the pathogenesis of atherosclerosis and its complications. CRP, CD40 ligand (CD40L), IL-18, monocyte chemotactic protein 1 (MCP1), among others, are inflammatory markers that result in endothelial activation.[68] There is also strong scientific evidence that inflammatory activation is an important pathway involved in the development and progression of atherosclerosis. The inflammatory cascade is involved in the entire process of plaque formation, from the early stages of endothelial dysfunction to the development of acute coronary syndromes.[69]

Interleukin-18 (IL-18)

IL-18, a member of the IL-1 cytokine family, is highly expressed in atherosclerotic plaques and found mainly in macrophages. IL-18 promotes the increase of adhesion molecule expression in vascular endothelium and, for this reason, increased IL-18 expression is directly associated with the development of endothelial dysfunction.[68]

C-reactive protein (CRP)

CRP is a circulating pentraxin consisting of five identical subunits arranged as a cyclic pentamer that has an important role on human innate immune response.[70] Research of the last twenty years have suggested that CRP may have proatherosclerotic effect, and directly affect adhesion molecule expression and fibrinolysis, thereby participating in inflammatory process of endothelial cells and development of endothelial dysfuntion.[71-73]

These studies have demonstrated that CRP promotes endothelial activation by expression of ICAM-1, VCAM-1, E-selectins and MCP-1, and activates macrophages to express cytokine and tissue factor, leading to enhanced LDL uptake by other lipoproteins.[73] In addition, CRP negatively modulates the production of endothelium-derived vasoactive factors, especially NO, which is the main regulator of vascular homeostasis. This process may facilitate endothelial cells apoptosis and attenuate important compensatory mechanisms to angiogenesis.[71]

Besides, CRP may stimulate the production of endothelin-1 (ET-1), a powerful endothelium-dependent vasoconstrictor, and IL-6, a key proinflammatory cytokine.[72] These data corroborate the hypothesis that CRP is a predictor of atherosclerosis and vascular death. CRP serum concentrations may reflect endothelial health, since this protein has the potential to change endothelial cell phenotype, and contribute to lesion formation, plaque rupture and coronary thrombosis. Therefore, CRP acts not only as an inflammatory biomarker, but also as a mediator of vascular disease.[60]

CD40 ligand (CD40L)

CD40L, found either in free or soluble form, is a type II transmembrane protein belonging to TNF superfamily, involved in a pathophysiologic pathway closely related to inflammation and atherogenesis.[74] Most circulating CD40L is expressed in platelets, which are not only critical elements for homeostasis, but also have the potential to trigger an inflammatory response in the vascular wall.[75] Once activated, platelets promptly express CD40L, which, in turn, induces endothelial cells to secrete chemokines and to express adhesion molecules, thereby promoting the recruitment and extravasation of leukocytes at the site of injury.[76]

CD40L can be quickly released from the platelet membrane as a soluble form. Both forms have prothrombotic and proinflammatory effects, enhancing platelet activation and aggregation, and platelet-leukocyte and leukocyte-endothelium conjugation. CD40L also increases the release of reactive oxygen species (ROS) and nitrogen from stimulated platelets.[77] In summary, CD40L activation system promotes a chronic inflammatory state in the vascular wall, contributing to the development of endothelial dysfunction, atherogenesis and respective complications.[74]

Monocyte chemotactic protein 1 (MCP-1)

Monocytes adhered to the vascular wall due to the effect of cell adhesion molecules transmigrate from the endothelium into the tunica intima, the innermost layer of the arterial wall. This monocyte migration is mediated by a concentration gradient of MCP-1, via interaction with the monocyte receptor CCR2. In the arterial intima, monocytes develop into macrophages and begin to express scavenger receptors, such as SR-A, CD36, and LOX-1, that internalize modified lipoproteins. This process gives rise to macrophages or foam cells that act in lipid transportation, which characterize early atherosclerotic lesions.[78]

MCP-1 induces the recruitment of monocytes after endothelial lesion, and this lesion seems crucial for the expression of MCP-1 in adherent platelets. Therefore, MCP-1 expression after vascular lesion is a highly specialized mechanism and exerts a key role in vascular remodeling in injury. Measurement of plasma MCP-1 may reflect the degree of endothelial dysfunction.[78]

Circulating endothelial cells (CEC) and endothelial microparticles (EMP)

Endothelial function reflects the balance between vascular endothelial lesion and repair. Based on this relationship, measurement of mobilization of mature endothelial cells and derived microparticles has been performed to estimate the endothelial injury degree. Circulating endothelial cells (CEC) are detached in activation or loss of integrity of vascular endothelium, and may be measured by flow cytometry or fluorescence microscopy. Once endothelial apoptosis begins, there is an abrupt increase in calcium release from sarcoplasmic reticulum, changing the status of the resting cell membrane.[54] Data in the literature have suggested a direct relationship between increased CEC levels in peripheral circulation and the extent of endothelial lesion in patients with atherosclerotic disease and vascular inflammation.[78]

Endothelial microparticles (EMP) are small vesicles that are released from the membrane of activated or injured CEC cells in response to activation and/or apoptosis.[78] Once released from the cells of origin, EMP express on their surface adhesion molecules, enzymes, receptors, and constitutive antigens,[53] whose composition may characterize the endothelial

progenitor cells. Normally, there is a local, slight, reversible endothelial activation, i.e., when the endothelium in the basal state starts to express cytokines and adhesion molecules, triggering inflammatory mechanisms, and circulating EMP are rarely found. Increased EMP levels have been found in a variety of conditions associated with endothelial activation or apoptosis.[66] suggesting a direct relationship of EMP with thrombogenesis and atheroma plaque formation,[55] and involvement of these molecules in inflammation, vascular injury and angiogenesis.[54]

Myeloperoxidase (MPO) and Reactive oxygen species (ROS)

MPO, a member of the heme peroxidase superfamily, is an enzyme released from activated neutrophils, monocytes, and tissue macrophages, that catalyzes ROS formation.[60] In addition to its activity against infectious diseases, MPO has been also recognized for its role in the onset and progression of atherosclerosis.[7,79] MPO can bind to glycosaminoglycans in the vascular walls and impair the release of endothelium-derived NO leading to local endothelial function.[7]

Products of MPO activity, such as hypochlorous acid, tyrosyl radical and nitrogen dioxide reacts with hydrogen peroxide and may contribute to oxidative damages in lipids and proteins in the body. Therefore, literature corroborates the idea that MPO levels may predict the development of endothelial dysfunction and coronary artery disease. Low MPO levels and certain specific MPO polymorphisms have been described as cardioprotectors.[60,79]

Although oxidative stress may promote endothelial dysfunction by numerous mechanisms, the main pathway involves the reduction in NO bioavailability. In certain circumstances, a chronic ROS production may exceed enzymatic and non-enzymatic antioxidant capacity, leading to an increase in oxidized biomolecules and associated tissue damage. Evidence has suggested that oxidative stress has a central role in atherogenesis, probably via development and progression of endothelial dysfunction.[66]

Vascular endothelial growth factor (VEGF)

VEGF, also known as vascular permeability factor, is widely known for its role in angiogenesis, promoting the proliferation and migration of endothelial cells, and increasing vascular permeability. It is produced by different cell types, including endothelial cells. VEGF binds mainly to cell surface receptor tyrosine kinases named VEGFR-1, VEGFR-2 and VEGFR-3.[80] Concerning the assessment of endothelial function, VEGFR-2 stands out among these receptors due to its expression restricted to vascular endothelial cells. It also has an important role in cell migration, endothelium-dependent vasodilation and angiogenesis.

VEGFR-2 activation occurs by its binding to VEGF or by increased blood flow shear rate, which triggers a signaling cascade and consequent activation of the MEK-MAPK pathway (proliferation and migration), the PI3K–Akt pathway (survival), and the Src–eNOS pathway (permeability). In adults, VEGFR-2 is physiologically detectable in low amounts in the blood. In adverse conditions, such as tumoral growth, injury repair, and inflammatory diseases, VEGFR-2 participates in the local revascularization process.[80]

Others

Ox-LDLs are proinflammatory, immunogenic molecules that may affect a great variety of atherosclerotic processes, from early events, such as adhesion molecule expression and activation of the immune system, to late events, such as platelet aggregation and destabilization of the atherosclerotic plaque.[7] Ox-LDL is produced by oxidative processes during LDL migration through blood vessel walls, and has been suggested as a marker of endothelial dysfunction and atherogenesis.[68]

Another endothelial function marker is free fatty acids (FFAs). FFAs may enhance ROS levels by increased cytokine production in mononuclear cells. In addition, FFAs may induce the activation of proinflammatory NF-kB pathways in human endothelial cells. Because to these characteristics, FFAs are considered an early biomarker of endothelial lesion and atherosclerosis, with important implications for the prevention and treatment of cardiovascular diseases.[60]

Procoagulant consequences of endothelial activation can be measured by changes in the balance between tissue plasminogen activator (tPA) and its endogenous inhibitor, plasminogen activator inhibitor-1 (PAI-1). Von Willebrand factor, a glycoprotein extensively produced in activated endothelium, has an additional role in the activation of cells and platelets, and induction of coagulation. In addition, fibrinogen may also be considered an endothelial function biomarker. This glycoprotein, synthesized mainly in hepatic cells and megakaryocytes, may bind to glycoprotein (GP) IIb/IIIa surface proteins

and for bridges to platelets. Fibrinogen stimulates migration of smooth muscle cells and platelet aggregation and increases blood viscosity; hence, it is associated with development of atherosclerosis.[66]

Conclusion

Evidence has shown the importance of endothelial dysfunction for the development and progression of cardiovascular diseases. Numerous *in vivo* or *in vitro*, and invasive or non-invasive methods have been used for the investigation of endothelial function. Although these techniques have been widely used in the clinical research, these methods cannot be used for diagnostic purposes due to their invasiveness, high cost and difficult standardization. Therefore, further studies and investments in the field are suggested to make these methods applicable in the clinical practice and, hence, minimize public health problems related to cardiovascular diseases by means of an early diagnosis of endothelial dysfunction.

Limitations

The present study is a narrative review of the literature and, for this reason, does not present a well-established, reproducible methodology. Identification, selection, analysis and interpretation of the studies reviewed were left to the authors' discretion. However, it is worth highlighting that the aim of this review was to provide an update of current methods used for the assessment of endothelial dysfunction and to point out new perspectives in this field.

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Author contributions

Conception and design of the research: Storch AS, Mattos JD, Alves R, Galdino IS, Rocha HNM. Acquisition of data: Storch AS, Mattos JD, Alves R, Galdino IS, Rocha HNM. Analysis and interpretation of the data: Storch AS, Mattos JD, Alves R, Galdino IS, Rocha HNM. Writing of the manuscript: Storch AS, Mattos JD, Alves R, Galdino IS, Rocha HNM. Critical revision of the manuscript for intellectual content: Storch AS, Mattos JD, Alves R, Galdino IS, Rocha HNM.

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