Review Article

Another story of Human Atherosclerosis

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Abstract

In this opinion review, we aim at presenting an integrative picture of human atherothrombosis pathophysiology, taking into consideration its phylogenetic determinants and chronological aspects. We first discuss how systemic arterial pressure, inherited from the acquisition of peripheral resistances and conductance artery multi-embraachments throughout evolution, generates outward convection of plasma molecules and particles through the arterial wall and favors collision of circulating cells with the wall. We then present the various responses triggered by the accumulation of cholesterol and blood cells in the arterial wall, with a more particular focus on the role played by vSMCs in triggering those responses in the early stages of the atheromatous disease. The contribution of SMC phenotypic plasticity to the formation of foam cells, introploaqueferrerotacy, oxidative stress, and angiogenesis is notably outlined. The mechanisms of plaque evolution towards vulnerability, erosion, and rupture are also discussed. Finally, the relationships between local hemodynamics and atheroma evolution towards a given vascular pathology are illustrated by the presentation of vascular territory-specific diseases including coronary and carotid atherosclerosis, peripheral artery disease and abdominal aortic aneurysms.

ABBREVIATIONS


INTRODUCTION

Cardiovascular diseases (CVD) linked to atherothrombosis remain the main cause of mortality and for a large part of morbidity in human. Atherothrombolic pathobiology is specific to the arterial part of the circulatory system and its various clinical expressions are related to preferential localizations of atheroma development in the arterial tree. Among those "privileged" atheroma-prone regions, the most notable ones are the coronary, internal carotid, femoral arteries, and abdominal aorta, whose atherothrombotic lesions respectively causes myocardial infarction, ischemic stroke, peripheral arterial disease, and aneurysm. Nevertheless, there is evidence that atheroma can develop elsewhere, if not anywhere, in the arterial tree, but some localization likely remains totally asymptomatic for a longer period of time than in functionally vital arteries.

To date, endovascular surgery remains the main treatment for symptomatic and late-stage atherothrombotic diseases. Carotid endarterectomy, stenting, endovascular stent grafting are among the most common interventions for CVD. Medication is principally used for prevention of primary and secondary thrombotic events using anti-platelet therapy and for management of cholesterol levels by the mean of statins. Notably, the role of cholesterol in atherothrombosis disease has been a subject of repeated controversies in recent years. In that context, it might be worth recalling that cholesterol retention in the arterial wall is a key initiating event of atheromatous lesion formation. More than a century ago, Nikolai Anitschkov showed that feeding rabbits with pure cholesterol was sufficient to cause atherosclerosis [1]. This finding has been largely confirmed since this founding study. So, why the controversy? Most of the studies that contest a role for cholesterol in atherothrombotic diseases underscore the fact that cholesterol levels do not correlate with the occurrence of acute cardiovascular events. However, this vision of CVD through the narrow spectrum of their clinical expression – though clinically relevant - largely disregards their chronic nature and kinetics. Atherosclerosis is indeed initiated in the first years of life and

develops slowly over decades before it manifests through one of its various forms of clinical expression. Another notable potential source of confusion is that the term cholesterol is almost exclusively used to refer to LDL-derived cholesterol. It is now known that besides LDL, other sources of cholesterol and lipids significantly contribute to the formation and growth of the lipid core of atheromatous lesions. Membranes of RBCs [2], platelets [3], and of dead SMCs and leukocytes indeed represent important sources of plaque cholesterol [4].

The last 30 years have seen the emergence of a new paradigm. Atherosclerosis is now commonly presented as an inflammatory disease, whose initiation and progression would be driven by immune cells and pro-inflammatory cytokines. In this pathophysiological inflammatory scheme, retention of apoB-containing lipoproteins in the artery wall triggers leukocyte infiltration into the sub endothelial space. There, phagocytosis of lipoproteins by macrophages would promote foam cell formation associated with the production of various chemo attractants and pro-inflammatory cytokines. This self-maintained series of events would ultimately lead to unresolved inflammation and evolution towards the formation of vulnerable plaques characterized by enhanced macrophage and smooth muscle apoptosis and necrosis, defective efferocytosis, decreased extracellular matrix production, and collagen degradation by macrophage proteases. Remarkably, this dominant paradigm has been mostly fuelled by studies conducted in hypercholesterolemic mice, with one immune cell and/or inflammatory mediator replacing another as the new central player in the pathogenesis of atherosclerosis on a regular basis, year after year. It should be noted, however, that so far, anti-inflammatory strategies derived from studies conducted in hypercholesterolemic mice have poorly translated for treating human atherosclerosis [5,6]. Several non-exclusive hypotheses have been proposed to explain the failure of anti-inflammatory agents in clinical practice. These possible explanations include lack of specificity and/or possible off-target effects [7]. It might also be worth considering the largely disregarded though well-documented limits of mouse models of atherosclerosis [8], as well as the striking differences between men and mice when it comes to inflammatory processes [9-11]. For example, it is known that human and mice lesions show marked differences in time span of lesion development, as well as in plaque cell composition, especially in the early stages of the disease. In particular, whereas leukocytes and macrophages are found in abundance in early lesions of mice, they become prominent at later stages in human lesions. In humans, this “inflammatory switch” seems to be highly related to intraplaque neangiogenesis, which is recognized as a major source of intraplaque hemorrhage and leukocyte infiltration contributing to plaque vulnerability and rupture [12]. Thus, the nature and role of inflammatory mediators in atherosclerosis likely follow a species- and plaque stage-specific expression patterns that contribute to interspecies discrepancies. Nevertheless, despite these interspecies discrepancies, the fact that systemic lupus erythematosus is associated with precipitated atherosclerosis illustrates how inflammatory and immune mediators can significantly impact in lesion progression in humans [13].

In this opinion review, we aim at presenting a balanced and integrative picture of human atherothrombosis pathophysiology, taking into consideration its phylogenetic determinants and chronological aspects.

**Phylogenesis of hemodynamics and arterial wall topology**

As mentioned above, atherothrombosis is restricted to the arterial tree. This noteworthy feature underscores the importance of hemodynamics and vessel wall structure in the development of atherosclerosis. The role of hemodynamics in the pathogenesis of atherosclerosis is further exemplified by the observation that the pulmonary circulation, in which the arterial pressure is much lower than in the systemic circulation, is usually devoid of atheroma. In the systemic circulation, arterial bifurcations are preferential sites of atheroma development. Therefore, one could consider atheroma as a frailty of the high-pressure systemic circulation related to the resistive function of its arterial tree, the muscularisation of the left ventricle, and to its multibranched anatomy. The parallel resistive and branched arterial model includes conductance and resistance arteries and arterioles, whose functioning is directly or indirectly coupled to the organ energetic demand. This metabolic autonomisation of organ functions is a result of the circulatory system teleonomic evolution. The phylogenetic transition from an in-series, low-pressure, circulating system animated by an archaic heart like in fish, to a high-pressure arterial system with organ-regulated directional blood flow has been accompanied by the appearance of new physical constraints together with the evolution of the arterial wall structure.

In physiological conditions, the media is a vascular tissue, devoid of capillaries. As a direct consequence, the arterial wall is an immune privileged site, poorly accessible to leukocyte diapedesis, which usually takes place in post-capillary venules in relation to their specific structure and functions of their endothelium. Nevertheless, vSMCs are highly plastic [14] and can shift their phenotypes particularly in the context of atherothrombosis. The very same forces that provide the arterial wall with oxygen and nutrients are also responsible for the accumulation of cholesterol in the sub endothelial space of arteries, particularly in hot spots of impedance and endothelial disruption. The high affinity of apo B100, the protein moiety of LDL, for the negatively-charged sulfate or carboxylic acid groups of matrix glycosaminoglycans leads to their accumulation and retention within the arterial wall, especially at the proteoglycan-rich intima/media interface. The convection of blood-derived particles and/or cells through the arterial wall is an important trigger of vSMC phenotypic transitions and, more generally, of the various arterial wall responses that drive atherosclerosis development. VSMCs can endooyte and phagocyte different particles and molecular complexes including LDL [15], protease-antiprotease complexes [16], apoptotic bodies [17], or senescent red blood cells (RBC) [18]. Moreover, vSMC can acquire an osteoblastic phenotype, promoting calcification [19], as well as a pro-angiogenic [7] or pro-inflammatory phenotype, thus stimulating centripetal neo-vascularization, and adaptive immunity in the adventitia [4]. All of these changes in vSMCs phenotypes contribute at some point to the growth and evolution of atheromatous lesions.
Outward convection of soluble mediators [20]

As a consequence of peripheral arterial resistances, an interstitial radial hydraulic conductance, orthogonal to the longitudinal circulation, is generated through the wall, related to the gradient between the luminal arterial pressure (130-80 mmHg) and the intersitial pressure in the adventitia (10-30 mmHg). Soluble plasma microparticles, macromolecules such as lipoproteins, soluble agents and mediators are convected through the wall by this outward radial conductance. Convection of plasma molecules is dependent on both, prevailing hemodynamic conditions (driving force), including luminal pressure, pulsatility, blood stagnation, and wall permeability involving integrity or desintegration of endothelial barrier, ECM bio-availability and SMC contractile functions. These concepts have been largely documented from a theoretical point of view by fluid mechanics and experimentally by in vivo uptake of circulating molecular tracers, showing that convection dominates diffusion process in the arterial wall. Convection of soluble plasma components are positively dependent on blood pressure and wall permeability [21], but also dependent on flow: high laminar flow has a washing effect of plasma components, limiting their outward convection. Conversely blood stagnation and decrease in shear stress, enhance convection of plasma components [22]. This outward convection is also locally influenced by hot spot of impedance. This “local” interactions between convection intensity and changes in arterial wall geometry, related to dispersion of velocity vectors, have been recently mechanically conceptualized and measured as transverse Wall Shear Stress, which predominates in systolic peak flow, by the group of Peter Weinberg in London [23].

In fact all the plasma components are outwardly convected through the wall, interacting or not with its different layers, according to their chemical and physical properties which determine their affinity for wall components, their ability to be retained and to accumulate with time, or to be endocyted and cleared by SMC. Such outward transport of soluble mediators not only concerns elements from the plasma, but also soluble molecules that are generated and released within the aortic wall, from the intima, convected through to the media to the adventitia. In this concept there is no inward extracellular retro diffusion of mediators from the adventitia, but mediators outward convection could generate inward living cell conductances in response.

Collision of circulating cells

The interactions of circulating cells with the arterial wall or between themselves are mainly the result of blood flow and changes in arterial geometry like those encountered at physiological embrauchments, or in case of pathological stenosis (e.g. endoluminal protrusions due to early plaques) or dilatations. Changes in arterial geometry modify hemorheology: provoke flow turbulences and dispersion of velocity vectors, thus favoring the local formation of hotspots of impedance and the collision of blood cells with the wall [24,25]. Red blood cells (RBCs) and platelets are the most abundant blood cells and therefore represent the main blood elements entering in collision with the arterial wall in those regions where the biomechanical stress drives the formation of intimal tears and of small intimal hematomas. In a laminar rheological environment, RBC concentrate in the central axis (core) of the stream and expell platelets toward the endothelium [26]. When the flow becomes turbulent this property disappears. Collision of RBC with the wall is potentially a major factor of oxidation since RBC release hemoglobin and redox-active iron, a main catalyst of oxidation (Fenton and Haber-Weiss reactions) [27]. Collision of platelet with the vessel wall in areas of de-endothelialisation or loosened endothelial junctions leads to interactions between platelets and the subendothelial matrix [28]. Those interactions can cause platelet activation and aggregation which are associated with the release of numerous mediators from platelet granules including growth factors, antiproteases [PN-I for instance] [12], chemokines, and immunomodulatory factors. Thus, in addition to be sources of cholesterol through the cholesterol contained in their plasma membrane, RBC and platelets promote oxidation and remodeling within the arterial wall. Endothelial breaches and entry points for RBC, platelets, LDL and other blood-derived factors can be healed and consolidated by vSMC proliferation and ECM synthesis. This healing process likely contribute to the progressive formation of the fibrous cap that eventually covers the atheromatous core.

Fatty streaks

Canonically atheroma process is initiated by the convection of plasma lipid transporters through the arterial wall and the specific interaction of apoB with the intimal proteoglycans secreted by vSMC, leading to LDL accumulation in the subendothelial layer [29]. LDL is conveyor of cholesterol and phospholipids. Beside LDL, Lp(a) is also a LDL-like molecule, characterized by the presence of an apo (a) covalently linked to one apoB. Apo(a) is specific of primates. Apo(a) is a plasminogen-like molecule, able to bind carboxy-terminal lysine via its KIV domain including binding to vascular cells through lysine-terminal membrane proteins [30]. This is potentially the mechanism by which Lp(a) is retained in the arterial wall. The plasma level of Lp(a) is genetically determined by the number of KIV repeats: more KIV repeats (1-10), higher is the Lp(a) concentration. Lp(a) is the major carrier of oxidized phospholipoids. Lp(a) plasma level is a pronostic marker of the CV risk, including calcifications [31].

Macroscopically, fatty streaks are identified as yellow bulges on the luminal smooth surface of the arterial wall (Figure 1). Microscopically, these lipid bulges develop on a cellular-rich background of vSMC, able to synthesize proteoglycans. These highly hydrophyllic proteoglycans could be easily visualize by Alcianblue oncryosections or fixed deparaffinized sections. The lipid component could be visualized by ORO on cryosections. On fixed deparaffinized section, lipids are eluted by xylène and toluene, two solvents able to dissolve paraffin but also tissue lipids. Therefore lipid areas appear as empty spaces separating nuclei under optical microscopy. Nevertheless ORO could be also visualized by red fluorescence at 550 nm w.l. which is more sensitive than optical microscopy. This lipid accumulation could be intracellular and extracellular. When they are observed intracellularly they define foam cells.

Foam cells

Foam cells are cells which have endocyted lipids. Intracellular lipids appear as large empty vacuoles within the cytosol of the intimal cells, but sometimes as more granulated cytoso.
These two aspects empty or granular, correspond to the initial description of translucid or electron dense cytosol in foam cells, using electron microscopy in human [32] and baboon fatty streaks [33]. The initial foam cells are essentially vSMC as characterized by electron microscopy or immunostaining of specific vSMC markers (α-actin, SMC myosin, transgelin), in the absence of CD45 marker (CD45 marker of cells of myeloid origin) [34]. All foam cells are CD68 positive whatever their origin (Figure 2). CD68 is a functional marker of endocytosis/phagocytosis associated with the fusion of cell phagosome and lysosome, and is expressed by vSMCs in response to lipid overload. CD68 expression defines vSMCs as endocytic or phagocytic cells. These observations support a potential switch of intimal vSMCs from contractile or synthetic phenotypes to a scavenger phenotype in the arterial wall in response to lipids retention. At this early stage, lipid can be visualized by ORO staining on cryosection. On deparaffinized (and thus delipidated) sections, fluorescence analysis of ORO staining can reveal some lipids associated with apoproteins, particularly fatty acids of phospholipids and lipids covalently linked to oxidized protein aggregates (ceroids). In non-delipidated sections, can be stained by bodipy (bodipyromethene) and filipin, which are compatible with aqueous staining recipes. These staining showed that in initial human lesions, cholesterol and lipid vesicles were mainly found in the extracellular space or as intracellular vesicles present in the vSMC cytosol [34]. This confirmed results from earlier studies using electron microscopy [35]. In polarized light microscopy, cholesterol is found in extracellular and intracellular spherical particles, whose birefringency is indicative of their liquid-crystalline nature (Reinitzer in 1888 in a letter to Lehmann).

**Early oxidation**

As mentioned above lipids could also be implicated in the initiation of ceroid formation. Ceroids (lipofuscin) are insoluble covalent aggregates of oxidized lipids and proteins forming polymeric pigmented (red heme) material easily detected by its autofluorescence at 550 nm, the w.l. of hemoglobin and hemeautofluorescence. Ceroid could correspond to small autofluorescent granules or extracellular autofluorescent rings (extension of the polymerization) [4]. At the initial stages of fatty streaks, ceroids are mainly granular. Presence of ceroids provides evidence of an intense phenomenon of oxidation able to covalently bind lipids and proteins. Therefore fatty streaks and subjacent foam cells are early sites of oxidation initiation in human atheroma. In a recent study we explored the source of this oxidation process, and identified redox-active iron as a powerful component of granular foam cells. For this purpose, we used the Perls reaction (ferrocyanid precipitation) for detecting Fe ++, usually bound to intracellular ferritin, as reserve form of iron, and Perls reaction followed by DAB oxidative polymerization for detection of Fe ++ forming redox-active couple with Fe +++ [36-38]. This method has two major advantages: high sensitivity for detecting iron as compared to Perls alone, and ability to detect redox-active couples of ionized Fe. By this method (Perls alone and DAB alone used as negative controls) we identified granular foam cells as an important site of redox-iron storage [27] (Figure 2). In this context redox-active Fe ++/Fe +++ couple is a powerful catalyst of oxidation, increasing 5-10 fold the activity of endogenous oxidases. We identified further hemoglobin and RBC as potentially the main source of Fe ++ in the intima of the arteries in human [27].

**Early proteolysis**

Since plasmin is able to enzymatically modify lipoproteins in atheroma [39], we also recently explored the metabolism of outward convected plasma-borne plasminogen and its ability to be converted in active plasmin in fatty streaks [16]. This conversion take place on a membrane platform of vSMC [40] constituted by S100A/annexinA2 heterotetramer exposing lysine residue. Beside t-PA, a role for urokinase is not excluded. We also reported the overexpression of the PN-1 serpin in vSMC and its ability to form tissue protease/antiprotease complexes, which can be endocyted by LRP-1, a scavenger receptor present on vSMC. One part of this PN-1 comes from aggregated luminal platelets. For the first time,
this study described a clearance function of vSMC for protease/antiprotease complexes generated by plasma protein convection in early human atheroma. This observation fits well with the study of Boucher et al. describing an enhancement of atheroma progression associated with the knock-out of LRP-1 in apo E−/− mice [41].

**Initial plaques (Fibro-atheroma)**

In direct or indirect responses to initial intimal lipidic, platelet, oxydative or proteolytic injuries, the medial vSMC, alerted by convected mediators, including growth factor as PDGF, could inwardly migrate towards the luminal surface progressively recovering the fatty streaks, forming an intimal, collagen and proteoglycan-rich, fibromuscular cap between the luminal and the lipid core [42]. Contrasting with fatty streaks and the fibromuscular cap, vSMC progressively disappeared within the lipid core, releasing cholesterol from dying foam cells, forming solid cholesterol crystals, easily recognizable as extracellular crystal cleft on fixed, deparrafined, histological sections. These clefts are usually associated with extracellular ceroid rings, linked to oxydation, which could related, at least in part, to redox active iron (Perls+DAB). The shoulder is usually the most biologically-active area of the plaque. Extracellular DNA and histones, less or more associated with cytosolic proteins from SMC (α-actin, myosin, transgelin), are frequently observed in the shoulders, providing evidence of SMC death. The shoulder could be also a site of hydroxypapitte crystal formation, related to the ability of phosphate-rich free DNA to precipitate calcium [43].

**Mechanism of aggravation: néo-angiogenesis**

In parallel to the initial plaque development, in relation with lipid accumulation, particularly with the fact that lipoproteins do not convey only cholesterol, but also phospholipids, these phospholipids could be metabolized by phospholipases, releasing separately both the polar hydrophilic phosphated head and the hydrophobic fatty acid part. Arachidonic acid is one of these fatty acids, able to initiate a metabolic cascade of intermediate signaling pathways, involving cyclo-oxygenase and prostaglandin synthesis. These signaling molecules generated from lipids, will be convected towards medial SMC. We recently demonstrated [7] that outwardly convected lipids mediators, particularly PGJ2, the endogeneous ligand of the PPAR-γ was able to activate medial vSMC to synthesize and secrete more VEGF [7]. Secreted VEGF will be outwardly convected from the media, initiating sprouting of endothelial cells within the adventitia, provoking inward neoangiogenesis [20]. Therefore inward cellular conductances (neoangiogenesis) respond to a lipid-dependent increase in outward convection of soluble angiogenic growth factors. This effect could be reproducing by rosiglitazone an agonist of PPAR-γ and prevented by PPAR-γ inhibitor GW9662. These human data fit well with an earlier work in apoE−/− mice, showing that adventitial neo-angiogenesis initiated in the radial outward projection of the...
lipid plaque [44]. These data also underline the importance of both, the principle of outward percolation of soluble mediators through the wall, and that the layered topology of the arterial wall is a physiological parameter in the arterial wall to never forget in pathology (see introduction). In the context of human atherothrombotic disease, neo-angiogenesis is the most important step, shifting the initial stages, fatty streaks and fibro-atheroma, to more complex pathology, involving intraplaque hemorrhages (IPHs), the progression toward vulnerable plaque and the clinical expression of the disease. Since neo-angiogenesis involves arterioles, capillaries and venules, neo-angiogenesis is permissive of leukocyte diapedesis, losing the immune privilege of the wall, involving extravasation of monocytes forming macrophages, highly active in phagocytosis, neutrophils and lymphocytes, extravasating from postcapillary venules, involved in immune response [45].

**Intraplaque hemorrhages**

Beside to blood-borne intraparietal hematoma, generated by collision of blood particulate elements with the wall, IPHs, related to the immaturity of intraplaque neo-angiogenesis [46], are an important cause of plaque vulnerability [12]. IPHs, causes and consequences, have been extensively reviewed [4]. Important points are that 1) IPHs are potentially repeated events, not only one, 2) it is usually difficult to date IPHs, since vulnerable plaques could associate recent and older events, 3) advanced necrotic core are usually transformed « hemorrhagic » core, because 4) old IPHs are difficult to recognize since they are metabolically transformed in a crushed biological mixture at different stages associating high oxidative and proteolytic powers, lipids and proteins, and micro or macro calcifications. Since RBC membrane is rich in cholesterol and phospholipids, IPHs enrich the hemorrhagic core with lipids and cholesterol clefts. RBC content is mainly present as iron-rich hemoglobin. Release of heme and redox-active ferrous iron is a powerful catalyst of oxidative activities through Haber-Weiss and Fenton reaction (see above). IPHs are also rich in blood-borne platelets, fibrinogenesis and fibrinolysis (plasminogen activation). IPHs also convey blood-borne neutrophils, activated in this environment, potentially forming neutrophil extracellular traps (NETs), and releasing oxidative activities (myeloperoxidase, MPO) and numerous proteolytic activities, including both serine-proteases (leukocyte elastase, cathepsin G, u-PA) and metalloproteinase such as MMP-8 & -9 [47]. In this context, oxidative and proteolytic activities associated with IPHs are potentially the main biological determinants of vulnerability. They injure the wall, including both the media and the fibro cellular cap, by different ways provoking plaque rupture. IPHs are site of active phagocytosis in order to clear and heal the hematoma. This phagocyte activity is easily detected by ferric iron storage (Perls reaction) in CD68+ cells. Both resident SMCs and diapedesis of monocytes/macrophages, from myeloid origin, through intraplaque neo-capillaries and venules, could assume these phagocyte functions.
HDL oxidation

Low level of circulating HDL is considered as a marker of atherothrombotic evolution [48]. It is particularly true in abdominal aortic aneurysm (AAA) [49] and leg PAD [50]. It has been recently demonstrated that HDLs are oxidized by MPO during their percolation through the atherothrombotic arterial wall, which induces their apo-A1 and favors its cross linking [51]. Whether the oxidation of HDL is only due to MPO remains to be explored. As an alternative oxidation pathway, Aslehe et al. [52] showed that hemoglobin-haptoglobin complexes could play a predominant role in HDL oxidation. This strongly suggests that heme/iron could have an impact on HDL oxidation. The hypothesis is that apo-A1 dissociates from the lipid core during the HDL outward transport trough the oxidative wall. Free apo-A1 would then be filtrated and degraded in the kidney [53] leading to decreased levels of circulating atheroprotective HDL. Contrasting with LDL, HDL is not retained and does not injure the wall, but the oxidative wall injures the HDL.

Calcifications

The process of intimal microcalcification takes place early in human atheroma, usually associated with initial plaque development. It could be associated with micro calcifications within the media (mediocalcosis). In all other stages macro or micro calcifications are constantly present in atherosclerotic lesions [54]. Therefore calcium score has a predictive value in atherosclerosis development and of clinical expression risk.

These classical data demonstrate the potential interactions of calcium with DNA phosphates in vitro. More recently Calcium was used to purify DNA colocalization between arterial cell-free DNA (DAPI, anti-DNA antibody, TUNEL, polylsine). Sometimes this colocalization could be evidenced on fixed non-treated histological sections, but sometimes the calcification itself impedes the direct recognition and necessitates pre-treatment of the section by EDTA, a chelator of divalent cations, including Ca++ (but also Fe, Zn, etc). We identified carotid, femoral, aorta, abdominal aortic aneurysms (AAA) as atherosclerotic sites in which calcifications could colocalize with tissue cfDNA. Similar studies on coronary arteries are in progress. We also infused purified DNA in the aorta of rats, and calcifications detected by microScan specifically appeared in the infused areas, but not in the non-infused areas. Nevertheless other organic phosphates are potentially source of inorganic ones. Phospholipid-rich lipoproteins metabolism within the tissue, could be also sources of phosphates, in particular Lp(a). The mechanism by which Lp(a) promote calcifications is not yet completely understood. Proudfoot and colleagues reported that phospholipid-rich microparticles or apoptotic bodies [58] released by vSMC are important mediators of calcification in the arterial wall. These results have been recently confirmed and extended to SMC exosomes and progression of calcification nuclei [59]. The Calcium Phosphate-rich hydrophobic exosomeprecipitated on collagen structure and accumulated in the tissue. In later stages vSMC could shift their phenotype to an osteoblastic one [19]. Different signaling is involved in this shift, phosphates, Runx2, BoneMorphogenic Protein, TRPM, KLF-4, etc.

Adventitial immune response

Because atherothrombotic injury is initiated at the interface with circulating blood by outward convection and intimal retention of LDL, most studies performed to unravel the mechanisms involved in injury-induced arterial responses have been focused on the innermost intimal layer. Nevertheless, as described above, small particles and soluble mediators are outwardly conveyed by hydraulic conductance throughout the arterial wall toward the adventitia. In atheromatous arteries, neoantigens are generated within the arterial wall, notably by oxidation and proteolysis, and a variety of mediators are secreted by plaque cells and by medial VSMCs underneath intimal lesions. Like plasma-borne factors, those neoantigens and plaque cells-derived mediators can reach the adventitia where they trigger various responses, angiogenesis being only one example of those responses. Advanced atherosclerosis is accompanied by the periodentinal formation of ATLO resembling germinal centers of lymph nodes. The presence of ATLO thus indicates that adaptive immune responses are also triggered by convected neoantigens and plaque or medial cell-derived mediators.

Although it remains unclear whether ATLO are protective or deleterious, there is already evidence that they might not be just passive bystanders associated with tissue injury. Indeed, the formation of TLO often marks a turning point in disease evolution. In alloimmune responses, TLO promote the antibody-mediated destruction of grafts. In autoimmune diseases, TLOs are clearly detrimental, promoting autoantibody production and activating cellular effectors, both of which result in organ damage [60]. Gräbner et al. [61], demonstrated that ATLO size correlated with plaque size and that ATLO always developed in atherosclerotic arterial segments in ApoE-/- mice. The role of B cells in ATLO is currently debated. It has been proposed that T-independent innate-like B cells and the natural antibodies they produce might be endowed with atheroprotective properties, whereas T-dependent B cells might have a proatherogenic potential [4,60]. The mechanisms and impact of adventitial immunity on arterial wall biology remains puzzling and unresolved but, just like angiogenesis from adventitial vasa vasorum, the formation of ATLO nicely illustrates how the initial intimal injury spreads and eventually affects the arterial wall in its entirety.

Specific forms and localizations

Coronary circulation and plaque rupture versus erosion: Hemodynamic of coronary arterial circulation is highly specific and different of the systemic hemodynamic. In systemic arterial circulation the peaks of flow and pressure are systolic and approximately concomitant. In contrast, the coronary circulation is different: there is no inflow in systole,
only venous outflow. Conversely the arterial inflow is maximal in protodiastole. This is due to the compression exercised by the myocardial contraction on the intramyocardial arterial circulation. The compressive force generated by systolic contraction is necessary more powerful than the driving force generated by arterial systolic pressure in the coronary artery. The intramyocardial circulation is like a sponge, compressed during systole, completely preventing incoming flow, but provoking venous outgoing flow, whereas relaxing diastole provoke high inflow [62]. This phasic circulation is specific to the intramyocardial compartment, whereas the epicardial coronary arteries remain out of this compartment, submitted to blood stagnation during systole and sometimes to reverse flow creating oscillatory flow in systole. Diastolic inflow provokes a high shear in stress in epicardial arteries. This hemodynamic specificity is probably the reason of the preferential localization of atheroma in epicardial coronary arteries. In systole, the highly pressurized stagnation of blood enhance the convection of lipoproteins from the plasma to the wall, initiating atheroma with fatty streak, fibroatheroma, angiogenesis and lastly intraplaque hemorrhages and plaque rupture. In addition, high diastolic shear stress potentially enhances the risk of erosion on already existing fibrous plaque (fibro-atheroma). Erosion-provoked thrombosis is a specific phenomenon observed in coronary epicardial arteries due high diastolic shear stress, particularly of tranverse WSS [63], on early fibro-atheroma. In contrast, erosion-provoked thrombosis is rarely observed in other atherothrombotic-prone territories as carotid for example.

**Stent neo-atherothrombosis:** In-stent neoatherothrombosis has been identified as a phenomenon which may participate to both in-stent restenosis and in-stent occlusion [64]. It corresponds to the delayed apparition, within the arterial tissue in close proximity to stent struts, of lesions which share some histopathologic characteristics with primary atherothrombosis [65]. They appear more frequently (64% vs. 31%) and earlier (two years after implantation 29% vs. 0%) within DES as compared to BMS [64]. Recent clinical studies using optical coherence tomography (OCT) suggest that neoatherothrombosis may participate to (very) late stent thrombosis. To date, the pathophysiology underlying this new disease entity remains unclear. However, it is well established that intramura red blood cells (RBC) stimulate atherothrombosis via an accumulation of membrane cholesterol and an oxidative activity catalyzed by hemoglobin/iron release [2,12]. We developed an observational approach on human coronary stents, obtained from explanted hearts in patients undergoing heart transplantation. In addition, we used an in silico biomechanical approach to evaluate the compliance mismatch between the rigid struts and the distensible arterial wall during the cardiac cycle [66]. In all stent samples, neoatherothrombosis was detected, particularly in peristrut areas. It consisted of foam cells and cholesterol clefts, with or without calcification. Iron and glycophorin-A were present in peristrut areas, as well as autofluorescent ceroids. Moreover, in response to neoatherothrombosis, an immune granuloma, had developed in the adventitia. Some of these observations could be reproduced in an experimental carotid stenting model in rabbits fed a high cholesterol diet. Finally, in silico models were used to evaluate the compliance mismatch between the rigid struts and the distensible arterial wall, using finite element analysis. They show that stenting approximately doubles the local distortion stress between the metallic stent and the remaining soft arterial tissue (von Mises stress, compliance mismatch) in the intimal layer. This observational and experimental study highlights peri-strut hemorrhages as the cause of in-stent neoatherothrombosis. The interaction between the metallic strut and the soft arterial wall, during cardiac movements, is the proposed mechanism [66].

**Carotid**

Culprit lesion of internal carotid leading to emboles and stroke are characterized by the pathway described above, involving neo-angiogenesis and intraplaque hemorrhages (see above) [4]. Erosion is not observed in carotid culprit plaques. In this context proteolysis and oxidation are the main determinants of vulnerability. As a peripheral artery the peaks of flow and pressure are systolic and concomitant in carotid. The development of carotid culprit lesion is site and hemodynamic specific. Usually the culprit lesion develops on the wall of the bulb of internal carotid. Some studies showed that vortex turbulent flow is physiologically present in bulb [67], five millimeters downstream the bifurcation. Therefore the laminar flow in the common carotid becomes turbulent and vertical in the bulb, with low wall shear stress, highly enhancing the development of initial plaque and their evolution to vulnerability. Presence of flow turbulence may damage the endothelial cells and provoke collision between cellular components of blood (platelets, RBCs and leukocytes) favoring the formation of luminal thromb, and emboles.

**Femoral and leg arteries**

PAD is also a frequent localization of atherothrombotic disease. PAD provides evidence of the diffuse character of the atherothrombotic disease, since PAD has a high risk value for Myocardial Infarction and Stroke. Usually the atherosclerotic lesion develops on the posterior surface of aorta, iliac and femoral arteries. One the characteristic of atheroma in femoral artery is the predominance of calcifications as compared to other sites [68]. These pathological data are associated with significant difference in OPG/RANK/RANKL triad expression in the two sites, carotid versus femoral [69]. Femoral calcifications, budding in the arterial lumen also retain DNA and hemoglobin [43]. The intraluminal budding of calcifications could be related to an initial luminal protrusion of calcium phosphate crystals on which circulating free DNA could bind, causing calcium phosphate to precipitate, forming a vicious circle of DNA/calcium phosphate precipitation [70].

**Acquired AAA**

The abdominal aorta is a site particularly sensitive to the development of atheroma, including initial fatty streaks and plaques. Due to the specific hemodynamics of the terminal aorta (reflexion waves on the iliac bifurcation), atheroma becomes rapidly circular, generating numerous asymptomatic plaque ruptures and formation of intramural clots which may, or may not, be healed by intimal fibro cellular cap formation. These mural thrombotic events potentially initiate the formation of a non-occlusive ILT [12], which becomes the main source of proteases.
(plasminogen, elastase, MMP-9) and oxidative mediators which are outwardly convected across the media, directly or indirectly initiating the adventitial response. Abundance of autofluorescent ceroids in the wall provides evidence of these oxidative phenomena in AAA. The ILT progresses in relation to dilation-induced hemorheological changes (vortexing) in blood flow. As previously described, the ILT is a highly porous neotissue [71], with spatio-temporal biological dynamics including luminal biochemical transformation of the clot, and abluminalysis at its interface with the arterial wall. Such biological dynamics lead to a multilayered morphology of the ILT corresponding to the different stages of its transformation: due to the enrichment in intact RBCs the luminal layer is red, the intermediate layer is brown, corresponding to hemoglobin metabolism, and the abluminal layer is highly granular, providing evidence of fibrin degradation and oxidative re-aggregation (fibrinolytic fragments can be reaggregated by oxidative covalent links). In addition to RBCs, the luminal layer is rich in aggregated platelets providing a platform for prothrombin activation and fibrin formation. Due to P-selectin exposure by aggregated platelets, the luminal ILT recruits circulating leukocytes, mainly neutrophils, which degranulate and die subsequent to tissue retention, releasing numerous components including u-PA, L-elastase, and MMP-9 & -8. Neutrophil components are partially and temporarily retained within the ILT by the formation of Neutrophil Extracellular Traps (NETs), filamentous complexes of free DNA and histones, capable of retaining cytosolic molecules.

In addition to proteolysis, the ILT is also the main source of oxidation processes due to the powerful catalytic enhancement of oxidase activities, mainly myeloperoxidase of neutrophil origin, and by heme-derived redox-active iron released by RBC degradation in the luminal layer [72]. In this study we reported that internal consumption of circulating RBC by the ILT, could lead to relative peripheral anemia, and iron retention, associated to AAA poor clinical outcome. Luminal RBC membranes also release unesterified cholesterol, which usually accumulates at the ILT/wall interface as solid cholesterol crystals (cholesterol cleft on deparaffinizedformol fixed sections). The presence of large amounts of iron in the adventitia (Perl’s reaction), provides evidence of RBC/heme degradation in the ILT, with subsequent iron release, outward transport to the adventitia and endocytosis by phagocytes. Moreover, plasma components, including HDL, which percolate through the ILT [72] can be oxidized during this mass transport, leading to decrease in circulating HDL [49]. Due to tissue activation processes, the luminal layer of the ILT releases a large amount of micro vesicles/particles of platelet, neutrophil and RBC origin, able to outwardly convect numerous membrane-bound mediators such as exposed procoagulantphosphatidyl-serines, components of the fibrinolytic system, ADAM metalloproteinases, etc. The ILT may be also a site for deep bleeding, acutely enhancing ILT proteolytic activities. The outward convection of proteases causes injury to the medial layer, leading to VSMC disappearance and breakdown of collagen and elastic fibers, allowing progressive dilation and, finally, ruptures.

The adventitial tissue of AAAs released immunoglobulins, thereby indicating that adventitial TLOs are functional B-cell structures. Strikingly, in the ATLO of AAA adventitia, the concentration of IL-21, a prototypic Th1 cytokine, was positively correlated with the diameter of the aneurysm, strongly suggesting that the Th1-dependent B-cell response is directly linked to the progression of the disease [4]. We believe that the topological organization of the arterial wall places the adventitia in a position where it concentrates most of the soluble/particulate mediators that percolate radially from the lesions, thereby resulting in a both high immune profile and convection of ILT-borne neo-antigens that promotes lymphoid neogenesis on this layer.

CONCLUSION

Atherosclerosis is a chronic pathology of the arterial wall, spontaneously occurring in human, due to the combination of with different risk factors, fostered by phylogenetically-determined specific hemodynamics. As a consequence of evaluative acquisition of peripheral arterial resistances, blood pressure initiates radially outward convection of plasma components, including lipoproteins through the wall. Outwardly convected LDL, and potentially LP(a), are retained and accumulated in the glycosaminoglycan-rich and SMC-rich intima, generating translucid foam cells. Fatty streaks could be enriched by RBC absorption and phagocytosis, generating granular foam cells. These fatty streaks are progressively recovered by a fibre cellular layer leading to the initial plaque (fibro-atheroma) in which the SMC progressively disappear of the extracellular lipid core. An essential step of disease progression at this stage is the development of an inward neo-angiogenesis which will later and progressively be responsible for intraplaque hemorrhages and plaque vulnerability at the end. At all stages of lesion development and progression, circulating RBC interactions with the wall play an important role as a pro-oxidant phenomenon. The specific topology of atherothrombosis development in the arterial wall is mainly related to hemodynamic, particularly to hot-spot of local impedance, flow dispersion and turbulence.

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