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REVIEW

Intraplaque haemorrhages as the trigger of plaque vulnerability

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Atherothrombosis remains one of the main causes of morbidity and mortality in the western countries. Human atherothrombotic disease begins early in life in relation to circulating lipid retention in the inner vascular wall. Risk factors enhance the progression towards clinical expression: dyslipidaemia, diabetes, smoking, hypertension, ageing, etc. The evolution from the initial lipid retention in the arterial wall to clinical events is a continuum of increasingly complex biological processes. Current strategies to fight the consequences of atherothrombosis are orientated either towards the promotion of a healthy life style[1] and preventive treatment of risk factors, or towards late interventional strategies.[2] Despite this therapeutic arsenal, the incidence of clinical events remains dramatically high,[3] dependent, at least in part, on the increasing frequency of type 2 diabetes and ageing. But some medical treatments, focusing only on prevention of the metabolic risk, have failed to reduce cardiovascular mortality, thus illustrating that our understanding of the pathophysiology of human atherothrombosis leading to clinical events remain incomplete. New paradigms are now emerging which may give rise to novel experimental strategies to improve therapeutic efficacy and prediction of disease progression. Recent studies strengthen the concept that the intraplaque neovascularization and bleeding (*Figure 1*, upper panel) are events that could play a major role in plaque progression and leucocyte infiltration, and may also serve as a measure of risk for the development of future events. The recent advances in our understanding of IntraPlaque Hemorrhage as a critical event in triggering acute clinical events have important implications for clinical research and possibly future clinical practice.

Keywords Angiogenesis • Cholesterol crystals • Haemoglobin • Protease • Adventitial tertiary lymphoid organs • Diabetes • Atherothrombosis

Early observational studies

Vulnerable plaques are characterized by the retention of highly modified, heterogeneous biomaterials within the core of the lesion. This so-called 'necrotic core' is encapsulated between the luminal fibrous cap and the outer intima and remaining media ('intramural atheromatous abscess' described by T. Leary in 1934).[4,5] The core includes components of different ages, cholesterol crystals, frequent calcific nodules, and a more or less identifiable fibrin-rich haemorrhages, highlighting the discontinuous evolution of the plaque from the initial esterified lipid retention to the formation of a more complex necrotic core, potentially responsible for plaque instability and complications.[5]

The complex nature of the necrotic core in plaques was first suggested by Galien (131 to 201 CE) in his initial description of human atheroma (αθηρωμα, atheroma = gruel). The involvement of the repeated accumulation of haemoglobin-rich intraplaque thrombi in the evolution of the lesions toward complications was proposed as early as 1936.[6] In these initial observational studies of human pathology, Paterson[7] and Wartman[8] described intraplaque haemorrhages caused by neo-capillary rupture (*Figure 2*, left panel) and claimed that fibrin-rich intraplaque haemorrhages are the common precipitating cause of arterial lumen thrombosis. After this initial period, the clinical and biological importance of intraplaque haemorrhages became rather neglected, and the majority of biological studies focused on lipid metabolism

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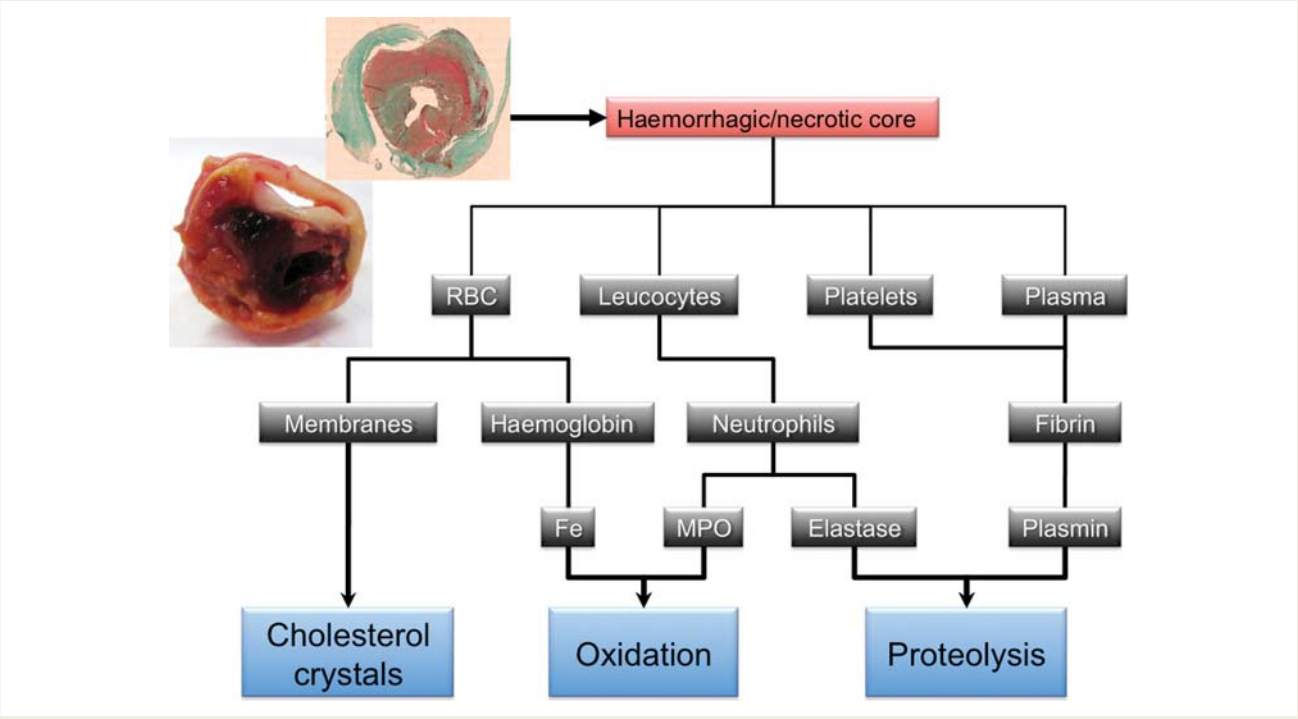

Figure 1 Macroscopic view and **s**chematic representation of the detrimental consequences of intraplaque haemorrhages on plaque biology and stability.

and on the inflammatory response mainly represented by leucocyte extravasation observed within the complicated plaque.[9]

Histopathological description

Histopathological studies are limited by the difficulties of precisely dating fibrin-rich intraplaque haemorrhages by histological examination due to the detersion of haemorrhagic products by phagocytes and progressive oxidative and proteolytic transformation. Virmani and Roberts[10] reported that the frequency of intraplaque, erythrocyte extravasation, and the presence of iron and fibrin were proportional to the number of atherothrombotic plaques present. Moreover, intraplaque iron and fibrin were mainly present in association with extravasated erythrocytes, suggesting that all the blood components of haemorrhages are present within the plaque, including plasma and cellular components. Recently, intraplaque haemorrhages have been observed macroscopically by the presence of red liquid in plaques (*Figure 1*, upper panel), identified microscopically by the presence of more or less intact erythrocytes (*Figure 3*) in the core of the plaque, or indirectly characterized by the presence of free haemoglobin, and iron staining with Prussian blue, observed as haemosiderin present in phagocytic cells (*Figure 2*, right panel). In the absence of intact erythrocytes, the presence of a large amount of iron provides evidence of older haemorrhages. Erythrocyte 'ghosts' can also be identified by the presence of glycophorin, an abundant antigenic protein of the erythrocyte membrane, detectable by immunohistochemistry[11] (*Figure 3D*). A recent autopsy study observed a higher density of neovessels in non-stenotic, human coronary plaques, which correlated with the presence of iron and glycophorin.[12]

Usually, intraplaque haemorrhages are associated with a high density of phagocytic cells (CD 68^{+}) involved in RBC and iron phagocytosis (*Figure 3C* and *Figure 4*, left panel). The clotting process takes place rapidly following intraplaque haemorrhage, involving platelet and thrombin activation and fibrin formation (*Figure 4*, right panel).

From intraplaque haemorrhages to clinical expression

Numerous clinical histopathological studies have been published since 1979, exploring the relationship between intraplaque haemorrhages in carotid endarteriectomy samples and clinical symptoms. In 1979, Imparato *et al.*[13] established a relationship between the presence of intraplaque haemorrhages in carotid endarteriectomy samples and neurological symptoms in a series of 50 patients. Following this initial study, there have been reports of a strong or weak association between macroscopic or microscopic intraplaque haemorrhages[14] and clinical events, which have been recently summarized.[15] Other studies focused on the relationship between intraplaque haemorrhages and neovessel density,[16,17] or on the prognostic value of carotid intraplaque haemorrhage as a predictor of global cardiovascular morbidity and mortality.[18] This prognostic value of

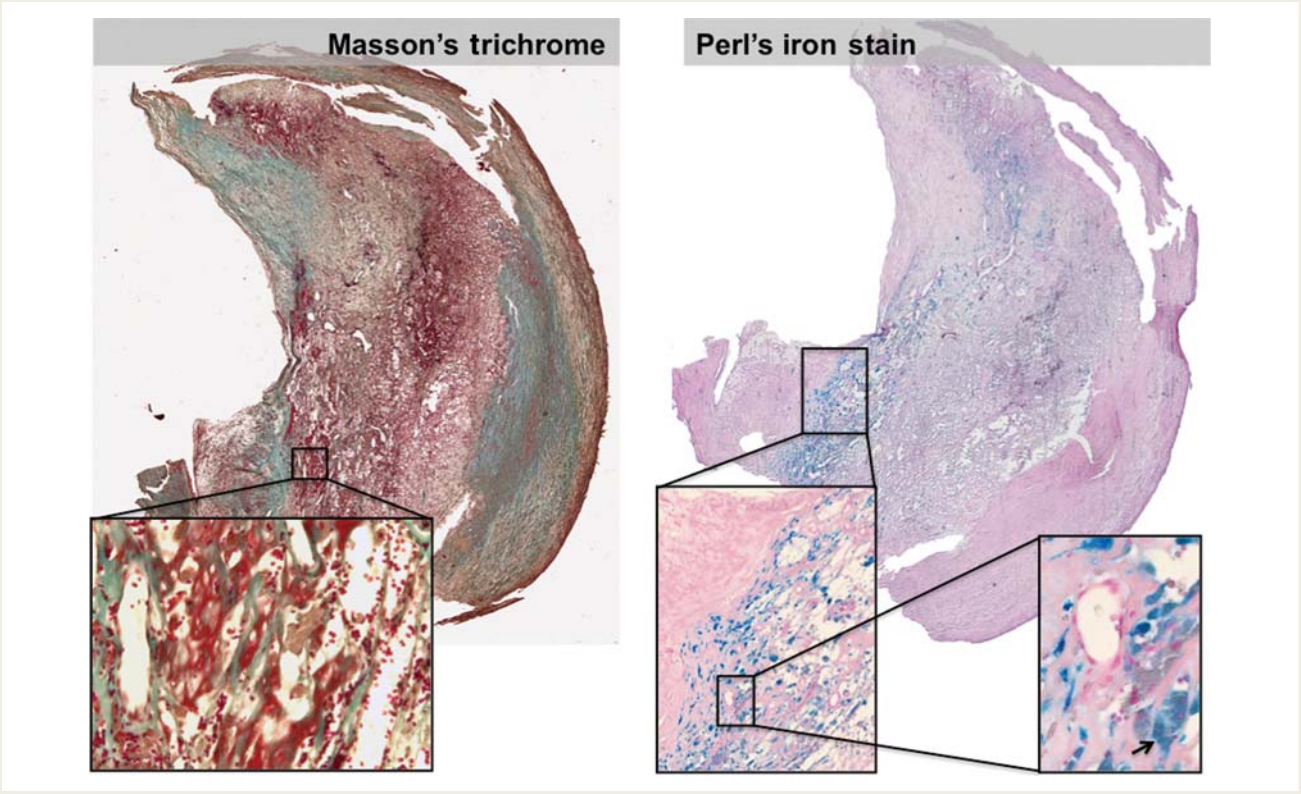

Figure 2 Histology of intraplaque haemorrhage (*A*: Masson's trichrome, mosaic reconstituted section, ×2.5) showing the presence of intact RBCs and free haemoglobin (inset, ×20). (*B*) Iron visualized by Prussian blue (Perl's) stain with nuclear red counterstaining, showing the haemoglobin-dependent presence of iron, mainly localized in the vicinity of neocapillaries and within phagocytic cells (insets, ×20 and ×40, arrow).

neovascularization and intraplaque haemorrhages has been recently emphasized through the Athero-Express biobank evaluation showing that local plaque haemorrhages and increased intraplaque neovessel density were independently related to cardiovascular outcome.[19] This study is of specific interest since it raised the concept that locally observed increased plaque neovascularization or haemorrhages are associated with an increased risk of secondary events in other vascular territories, suggesting that the degree of plaque vascularization and bleeding in one site may reflect the situation in other vascular sites.

From centripetal neo-angiogenesis to intraplaque haemorrhages

The mechanisms of plaque enrichment by blood-borne components has been a matter of debate between those favouring repeated plaque fissuring and associated formation of a non-occlusive luminal thrombus which is then incorporated into the plaque[20] and proponents of intraplaque haemorrhages being related to leakage from intra-plaque neo-capillaries.[21] The fact that erythrocyte extravasation and intraplaque haemorrhages could be observed in relation to a high density of neocapillaries in the absence of plaque fissure provides evidence of the predominant role of the former. Nevertheless, incorporation of luminal thrombi cannot be entirely excluded, particularly in large arteries. For example, it has been reported that atheroma development in pulmonary artery hypertension is directly linked to the migration, adhesion, and subsequent incorporation of mobilized venous thrombi within pulmonary artery wall.[22] Similarly, some incorporated luminal thrombi, unrelated to intraplaque haemorrhages, but resulting from plaque fissuring, can be observed in human aorta.

Intraplaque haemorrhages are mainly related to centripetal angiogenesis from the adventitia towards the plaque.[23] This neo-angiogenesis takes place early in atheroma development and is related to lipid overload. Heistad *et al.*[24] described an increased perfusion in the outer layer of the aorta of hypercholesterolaemic monkeys. Hypercholesterolaemia promotes the development of adventitial coronary vasa vasorum in a porcine model.[25] In this model, coronary neovascularization development preceded hypercholesterolaemia-induced endothelial dysfunction,[26] and may promote plaque progression.[27] Conversely, hypercholesterolaemia is associated with an elevated plasma level of VEGF in humans and statins reduced both hypercholesterolaemia and plasma VEGF concentration.[28] Centripetal neo-angiogenesis, erythrocyte extravasation, and erythrophagocytosis are early events in atheroma progression, usually not observed in association with fatty streaks, but constantly associated with the fibro-atheroma stage[29] (*Figure 4*).

These neo-capillaries could allow diffusion of plasma-borne molecules[30] and diapedesis of erythrocytes and leucocytes. Indeed, the

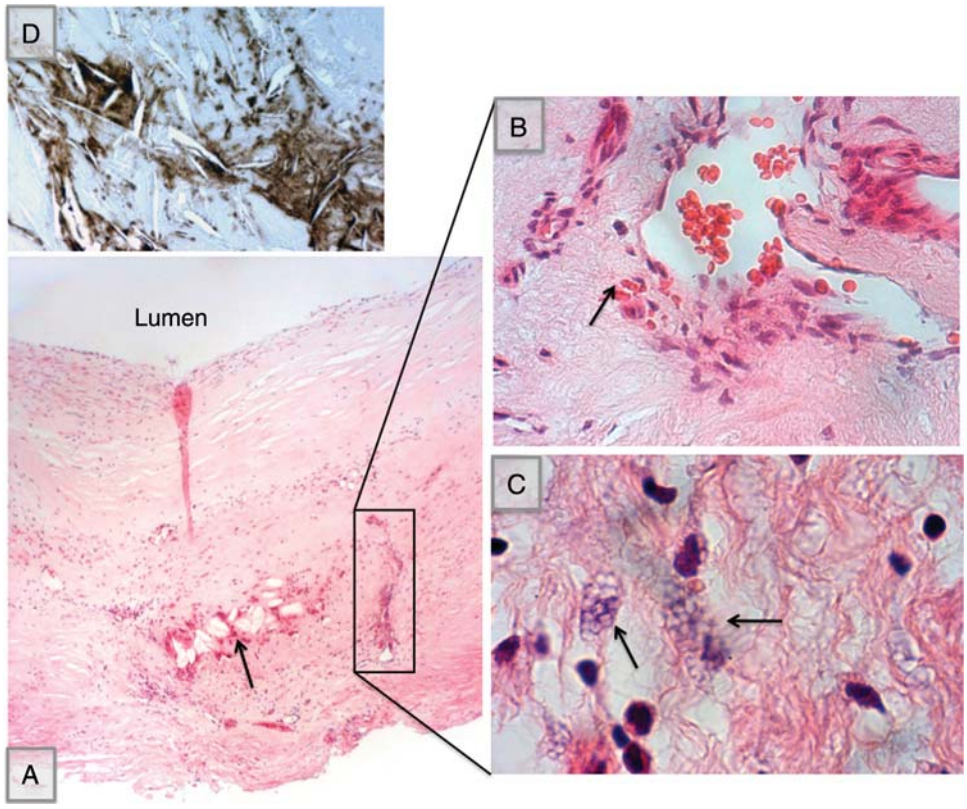

Figure 3 Centripetal angiogenesis and erythrodiapedesis in early stages of human atheroma. (*A*) General view of an asymptomatic lesion in human common carotid artery showing both cholesterol crystal clefts (arrow) and centripetal angiogenesis (square) (haematoxylin/eosin, HE ×4); (*B*) enlarged view of the neovessel showing the presence of RBCs within the circulating lumen, and erythrodiapedesis outside the vessel (arrow, HE ×40); (*C*) erythrophagocytosis (entosis) evidenced by the presence of RBC skeletons within a phagocyte (arrow, HE ×40); (*D*) enlargement of cholesterol crystal clefts surrounded by immuno-staining of glycophorin A (brown, ×40).

density of intimal neo-capillaries correlated with the extent of necrotic core formation, intraplaque haemorrhage, haemosiderin deposits, and inflammatory infiltrates,[31] suggesting that centripetal angiogenesis is a determinant of atherothrombotic evolution. Neovascularization has been reported in human plaques, whatever their localization: carotid arteries,[17,32,33] coronary arteries,[21] and aorta,[34] all correlating with plaque evolution. Therefore, adventitial neo-angiogenesis appears to be linked to the evolution of atherosclerosis from the early stages towards complicated lesions. The driving force of centripetal angiogenesis is the transmural convection, from inside to outside, due to orthogonal hydraulic conductance, of soluble mediators, such as VEGF, from the wall to the adventitia.[23]

The immaturity of neo-vessels is the cause of intraplaque haemorrhages occurring in their vicinity.[21] Indeed, neo-vessels in plaques appear to be leaky.[35,36] When localized in plaques, microvessels are dysmorphic and lack surrounding α-actin-positive mural cells.[31,37] It was recently observed that endothelial cells lining microvessels were abnormal, consisting of membrane blebs, vacuoles, open intercellular junctions, with a tendency to detachment.[38] These studies suggest that neovessel immaturity is directly involved in leucocyte and erythrocyte extravasation and therefore in intraplaque haemorrhages. Since the angiopoietin system plays a role in vessel maturation/destabilization, the balance between angiopoietin-1 and angiopoietin-2 expression was explored in human plaques.[39] A negative correlation was observed between angiopoietin-1 and microvascular density within the plaque, whereas the ratio angiopoietin-2/angiopoietin-1 was positively correlated to microvascular density. This imbalance has been linked to the proteolytic environment of the plaque, involved in the degradation of angiopoietin-1 and in Tie-2 receptor shedding.[40] Angiopoietin-1 promotes endothelial cell barrier integrity, and imbalance between angiopoietin-1 and angiopoietin-2 is thought to play a role in brain arteriovenous malformations,[41] which lead to recurrent cerebral haemorrhages. Also absence of pericyte recruitment and impaired vessel maturation is reported in myocardial ischaemia in diabetic mice with attenuation of Tie-2 and increase in angiopoietin-2 expressions.[42] Lastly, haem/iron, released by free haemoglobin, is directly toxic for endothelial cells.[43] Therefore, immaturity and fragility of vessels arising by intraplaque neo-angiogenesis may explain the commonly observed presence of erythrocyte extravasation and haemorrhages of different ages in one evolutive plaque[14] and the recurrence of intraplaque haemorrhages after a first haemorrhagic event.[44]

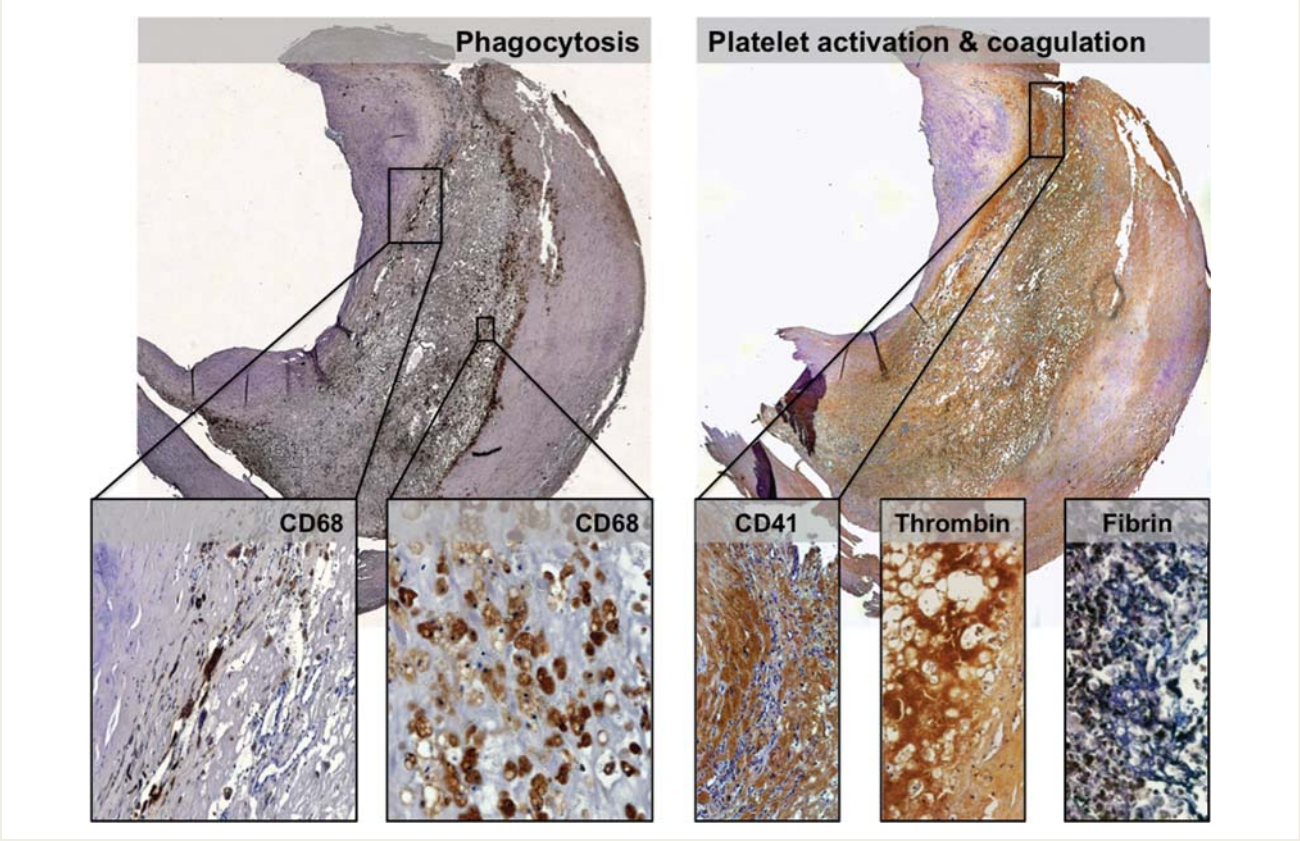

Figure 4 (Left panel) Immunostaining of phagocytes (CD 68) in the border area of the necrotic core (mosaic reconstituted section, ×2.5) showing CD 68^{+} smooth muscle-like cells (left inset, ×20) and phagocytosed RBCs (right inset, ×40). (Right panel) Immunostaining of platelets (C41) showing an important enrichment of the core by the platelet membrane marker (mosaic reconstituted section, ×2.5), particularly in the shoulder area, near the neovessels (×20). Thrombin immunostaining and phosphotungstic acid haematoxylin (PTAH) staining of fibrin are diffuse showing the fibrin-rich nature of intraplaque haemorrhages (×20).

Nevertheless, the further understandings the cellular and molecular events involved in centripetal angiogenesis and neovessel leakages remain an important scientific and medical challenge.

Biological consequences of intraplaque haemorrhages

Neoangiogenesis and its associated intraplaque haemorrhages convey into the lesion all the blood components, including red blood cells, leucocytes, platelets, and plasma proteins (*Figure 1*, lower panel). These different blood-borne components are implicated, to different degrees, in the biological processes involved in atherothrombosis progression, influencing mainly three predominant pathological aspects: cholesterol crystal production and retention, oxidant activities, and proteolytic activities within the lesion core.

Cholesterol crystal formation and retention

All stages of atherothrombosis are impacted by cholesterol, including cholesterol esters in the form of droplets, mainly conveyed by lipoproteins, and cholesterol crystals, composed of unesterified cholesterol, which usually remain localized within vessel wall (*Figure 3*). These crystals form the intracellular and extracellular clefts observed in histological section of fixed, paraffin-embedded tissues. The mobilization of cholesterol crystals from tissue into circulation is an infrequent but highly pathogenic phenomenon.[45] Free cholesterol retention in cells and tissues, leading to monohydrate crystal formation, can originate from endocytosed cholesterol esters, hydrolyzed in phagolysosomes,[46] or directly from free cholesterol of cell membranes. Membranes of circulating cells, including activated platelets,[47,48] and probably dead leucocytes, can release free cholesterol. But the cholesterol content of erythrocyte membranes exceeds that of all other cells in the body, with lipids constituting 40% of their weight.[49] Acyl coenzyme A:cholesterol acyltransferase inhibitors highly enhance cholesterol crystal formation by blocking cholesterol esterification and solubilization.[50]

The relationship between cholesterol crystals and erythrocyte membranes was first suggested by Arbustini *et al.*[22] who demonstrated that atheroma observed in pulmonary hypertension is only observed in thromboembolic pulmonary hypertension, and that pultaceous cores with cholesterol clefts are co-localized with glycophorin A immunostaining. This observation was rapidly extended to coronary atherothrombotic plaques.[51] The authors

observed that cholesterol crystal clefts co-localized with glycophorin A surrounded by iron deposits in human coronary plaques and were correlated with the complexity of the plaque. They also demonstrated experimentally that injection of erythrocytes into the arterial wall of hypercholesterolaemic rabbits induced cholesterol crystal formation and iron deposits. This experimental model has now been reproduced for exploring the relationship between erythrocyte accumulation and oxidative stress.[52,53] The pathogenicity of cholesterol crystals is linked to their ability to rupture biological membranes,[54] to erode the thin cap,[55] and to protrude within the lumen inducing luminal thrombosis[56] and possibly embolism. Lastly, cholesterol crystals within vascular cells at the early stage of atheroma could trigger the inflammatory response. Duewell *et al.*[57] and Rajamaki *et al.*[58] recently reported that intracellular cholesterol crystals, as other forms of crystals (uric acid, silica crystals, etc.), are able to activate NLRP3 inflammasomes through phagolysosome damage, cytosolic cathepsin release, and to induce pro-IL-1β cleavage and active Il-1β release into the extracellular space. Therefore, arterial wall cholesterol content is associated with arterial thrombosis.[59]

Haemoglobin and oxidative enzymes

Erythrocytes of intraplaque haemorrhages convey and rapidly release large amounts of haemoglobin (*Figure 2*, left panel). It has been reported that hydroperoxydes,[60] oxidized LDLs, and lipids extracted from human atheromatous plaques[61] are able to provoke RBC lysis. Haemoglobin is composed of a globin protein core and iron-containing haem (red pigment). Lipids extracted from atheromatous plaques can also oxidize Fe^{++} haemoglobin to the more reactive Fe^{+++} haemoglobin.[61] Fe^{+++} haem dissociates more easily from globin than Fe^{++}, releasing highly deleterious, hydrophobic-free haem/iron.[62] Haem/iron can mediate oxidative modification of lipids and cause endothelial cytotoxicity.[43,63] Therefore, haem/iron considerably amplifies the oxidative capacity of the biological systems, including the formation of protein complexes of high molecular mass, participating in the formation of the 'gruel' in the necrotic core. The haemorrhage-dependent colocalization of CD163 (haemoglobin scavenger receptor) and 4-hydroxy-2-nonenal, a marker of oxidation in human unstable coronary plaques, has recently been reported.[64] Histologically, Prussian blue (Perl's) staining usually reveals the presence of haemosiderin associated with phagocytes in the close vicinity of neocapillaries (*Figure 2*, right panel).

In parallel, haemorrhages convey neutrophils and mononuclear cells into the plaque, and leucocytes also extravasate from intraplaque neo-capillaries. Neutrophils are powerful pro-oxidative cells, due to their oxidative enzymes, NADPH oxidases, and myeloperoxidase (MPO), a haem protein abundantly expressed in neutrophils.[65] However, these enzymes are not totally specific: macrophages[66] also express MPO, but to a lesser extent than neutrophils.[67]

Conversely, in response to this iron-dependent oxidative environment, cells can counteract this oxidative injury through different anti-oxidative mechanisms operating at different stages of the process: RBC capture and entosis (cell dying as a result of becoming engulfed by a neighbouring cell) through a mechanism dependent on exposed phosphatidylserine,[68] ligation of free haemoglobin by haptoglobin and phagocytosis by CD163,[69] free haem and iron binding by haemopexin and endocytosis of the complex, and iron transport by ferritin. All these molecules limit the ability of free haemoglobin to generate oxidative stress. In particular, the haptoglobin genotype is a determinant of oxidative activity of free haemoglobin,[70] and of iron content[71] in human plaques.

Similarly, numerous cellular enzymatic or chelating molecules are present in complicated plaques and are involved in limiting the potential injury caused by the oxidative radicals generated. For example, thioredoxin has been reported to be more highly expressed in culprit coronary plaques than in stable plaques, in relation to intraplaque haemorrhages and iron deposits.[72] Their deficits are involved in atherothrombosis acceleration.[73]

Blood-borne proteolytic activity

The role of the mural haemoglobin-rich thrombus as an important source of proteolytic activity in atherothrombosis was first documented in human abdominal aortic aneurysm (AAA), an atherothrombotic pathology in which proteolysis plays a predominant role.[74] Similar blood-borne protease activities are generated during the evolution of intraplaque haemorrhages and play an important role in fibrous cap thinning and final rupture. Necrotic/haemorrhagic cores, associated with the risk of rupture, are characterized by fibrin deposits.[75] Neutrophil density is also a hallmark of plaque complexity, linked to both intraplaque haemorrhages and microvessel density[76] (*Figure 5*).

All the fibrinolytic activities, including t-PA, u-PA, and plasmin, are proportional to plaque complexity and are concentrated in the core of the lesion.[77] Similarly, neutrophil gelatinase and serine protease activities are mainly conveyed by bleeding within culprit plaques.[78] Beside their ability to degrade the extracellular matrix, these serine proteases can also degrade atheroprotective proteins secreted by smooth muscle cells, such as HSP.[79,80] Therefore, proteases conveyed by intraplaque haemorrhages could participate in the formation of the necrotic core gruel, and be a determinant of fibrous plaque fissuring and rupture.

However, platelets and angiogenesis-driven macrophage extravasation could also convey factors of resistance to proteolysis, such as PAI-1 and protease nexin-1.[81] Indeed, the role played by extravasated macrophages from neo-microvessels at the end-stage of plaque evolution is ambiguous, possibly involved more in detersion and healing the process, through M2 differentiation,[82,83] than in plaque rupture.

Adventitial immune response

The role of inflammatory cells, including lymphocytes, and various molecules involved in atherothrombosis have been recently reviewed.[84] The post-capillary venules are probably the predominant site of leucocyte diapedesis and subsequent movement towards the plaque, as they are specifically equipped for leucocyte rolling and transendothelial migration. Most of the intraplaque leucocytes observed in association with haemorrhages are macrophage-like phagocytic cells (*Figure 4*, left panel).

Intraplaque haemorrhages also impact the adventitial immune response. As in aortitis[85] and AAA,[23] complicated vulnerable

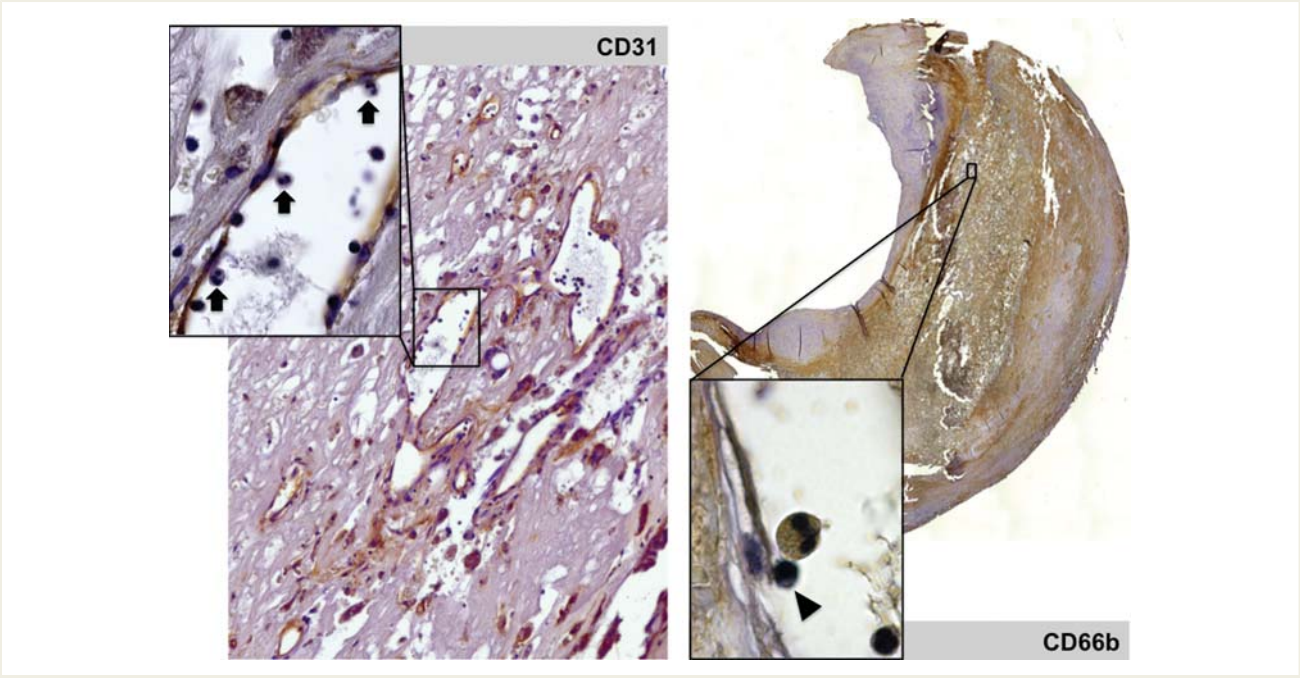

Figure 5 (Left panel) Immunostaining of endothelium (CD 31, ×20) showing the abundance of neovessels, and the presence of rolling polymorphonuclear cells within these neo-venules (inset, ×40, arrows). (Right panel) Diffuse immunostaining of neutrophil membranes (CD 66b, mosaic reconstituted section, ×2.5) and presence of a rolling CD 66^{+} polymorphonuclear cell and a mononucleate cell (arrow head) in a neovessel (inset, ×100).

plaques are characterized by the presence of an adventitial immune reaction, involving the formation of variable lymphoid nodules, possibly evolving towards adventitial tertiary lymphoid organs (ATLO). As early as 1985, Kohchi *et al.*[86] observed in autopsies that coronary lesions responsible for fatal unstable angina exhibited more adventitial lymphoid infiltrate, often associated with autonomic nerve fibres. Further details of this phenomenon have been reported, including the presence of undefined adventitial inflammation[87] and mast cells,[88] and the ratio between T and B lymphocytes.[89,90] These observations have been recently confirmed by reports of a spatial relationship between adventitial lymphoid infiltrate, plaque complexity, intraplaque haemorrhage, and luminal thrombus, and their association with hypertension,[91] suggesting a direct relationship between neo-mediators generated within the plaque, their orthogonal convection towards the adventitia, and their involvement in the adventitial immune response.[23] The formation of these lymphoid structures requires, and is associated with, an intense angiogenic process. Furthermore, the relationship, if any, between immune cell effectors recruited in ATLOs and those extravasating through intimal neocapillaries remains to be studied.

Impact of risk factors on intraplaque haemorrhages

Risk factors, downstream to their impact on metabolism, could influence intraplaque haemorrhage and its consequences. As previously stated, cholesterol-rich diet-induced adventitial and intramural neovascularization is attenuated by statin treatment, suggesting that cholesterol overload and lowering may affect vessel growth in atherosclerotic lesions. However, the influence of cholesterol on intraplaque bleeding needs to be established. Erythrocyte membranes are particularly rich in cholesterol, and hypercholesterolemia modifies RBC membrane content,[92] particularly in diabetes.[93] Therefore, membrane cholesterol levels of circulating erythrocytes have been recently proposed as biomarkers of atherothrombosis.[94] Similar results were reported for cholesterol density in neutrophil membranes and for neutrophil sensitivity to angiotensin II-induced free radical release.[95] In contrast, circulating HDLs could have beneficial effects via their capacity to convey α1-antitrypsin[96] into the diseased tissue, whereas tobacco consumption has an inverse impact.

When compared with data on intraplaque haemorrhages and neovessels in atherothrombosis, reports concerning the direct impact of diabetes and hyperglycaemia on this pathological process remain scarce. Drielsma *et al.*[97] reported that carotid plaques in diabetic patients are more highly vascularized than in non-diabetic patients and that control of hyperglycaemia reduced intraplaque neovascularization.[98] In apoE-deficient mice, induction of type 1 diabetes promotes more inflammatory and haemorrhagic plaques[99] but not neovascularization. Therefore, diabetes promotes microangiopathic neovessels in the plaque as it does in the retina,[100] whereas it impairs macroarteriogenesis in peripheral arterial disease.[101] Hyperglycaemia induces abnormal angiogenesis and micro-angiopathy in the retina via a VEGF-dependent mechanism.[102] Similar mechanisms could take place in the vascular wall at initial stages of atheroma, causing enhanced angiogenesis,

extravasation, and capillary fragility. At the stage of intraplaque haemorrhages, the impact of diabetes is mostly dependent on free haemoglobin-induced oxidative stress.

In remarkable studies, Levy *et al.*[69] demonstrated that the Hp1-1 genotype/phenotype of haptoglobin protects diabetic patients from cardiovascular complications. This beneficial effect is due to the greater ability of Hp1-1 haptoglobin to clear free haemoglobin via CD163. In contrast, the homozygous Hp2-2 genotype/phenotype does not facilitate haemoglobin clearance, and therefore the oxidative potential of free haemoglobin is reinforced in such patients.

Application to diagnostic imaging

Imaging atherothrombotic plaques is a large field of experimental and clinical investigation, widely reviewed in the international literature. The use of magnetic resonance imaging (MRI) for intraplaque haemorrhage has been recently reviewed.[103] In particular, MRI studies have documented that intraplaque haemorrhages are associated with plaque enlargement within 18 months, whereas without haemorrhage, atheromatous plaques did not progress.[44,104]

It may become also possible to visualize the vasa vasorum in atherosclerotic plaques and to follow their progression[105] using contrast-enhanced intra-vascular ultrasound imaging.[106] This could become of interest since vaso vasorum may be altered in response to plaque stabilizing compounds[107] and also neovascularization density of plaques has been correlated with adverse cardiovascular outcome in a longitudinal study.[19]

Circulating biomarkers that may reflect the risk of intraplaque bleeding

Today, there are more indirect than direct biomarkers of intraplaque microbleedings. The cholesterol concentration in erythrocyte membranes probably reflects the lipid profile over a long period of time. Tziakas *et al.*[94] reported that the non-esterified cholesterol content of circulating RBC membranes was strongly associated with clinical instability in patients with coronary artery disease.[108,109] This increase in cholesterol content was associated with a parallel increase in IL-8 RBC membrane (Duffy Antigen/Receptor for Chemokines, DARC) retention in patients with acute coronary syndrome.[109]

Conversely, plasma levels of anti-oxidant proteins could be used as biomarkers of RBC and free haemoglobin-dependent oxidative stress.[110]

Haptoglobin genotype/phenotype are also considered identifiable genetic risk factors of acute atherothrombotic clinical events in diabetic patients.[111] The homozygous Hp1-1 haplotype is protective, whereas the homozygous Hp2-2 or heterozygous Hp1-2 haplotypes are permissive.[69] Nevertheless, this powerful effect is restricted to diabetic patients.

In the same way, CD163, the Hb/Hp complex receptor, which is a transmembrane protein highly sensitive to proteolytic shedding and release, has been reported to be increased in the plasma of patients with peripheral artery disease.[112] A decrease in plasma levels of proteins secreted by smooth muscle cells[113] but degraded by blood-borne intra-tissue proteolytic enzymes,[80] or an increase in proteolytically generated peptides, could also be of interest as haemorrhage-dependent biomarkers. Nevertheless, the sensitivity and the specificity of these circulating markers with respect of intraplaque haemorrhages remain to be defined.

Therapeutic consequences

There is experimental evidence that statins preserve the adventitial vasa vasorum architecture and prevent neovascularization development in hypercholesterolaemic pigs, independently of cholesterol lowering.[114] Statins could also influence the consequences of microbleeding due to their ability to limit the cholesterol content of RBC membranes.[115] In particular, statins change the profile of cellular phospholipids by reducing the sphingomyelin content of cell membranes.[116] In parallel, statins are also able to limit neutrophil transendothelial migration.[117] In contrast, coumarin-type anticoagulation is associated with a higher occurrence of intraplaque haemorrhages.[118]

The interest in intraplaque angiogenesis has been spurred by the potential to target plaque neovascularization with angiogenesis inhibitors, including gene therapy and theranostic methods, approaches that have been associated with reductions in plaque progression in animal models. For example, angiostatin has been shown to limit plaque progression in mice.[119] More recently, it was shown experimentally that thalidomide, which impacts neomicrovessel formation, could prevent plaque progression in pigs.[107] Therefore, antiangiogenic therapy has been proposed in atherosclerosis.[120] Nevertheless, as described above, intraplaque angiogenesis has a dual role: neo-angiogenesis is responsible for the intrapalque haemorrhage itself, but also conveys leucocytes capable of detersion of haemorrhagic products, a necessary step towards healing. Indeed, there are now some recent clinical reports showing that antiangiogenic therapy for cancer[121] or age-related macular degeneration[122] could increase the risk of cardiovascular diseases. Conversely, rosiglitazone, a PPAR agonist, despite its beneficial effects on glucose metabolism, significantly increases the risk of atherothrombotic events.[123] It has been reported that PPAR agonists increase the expression of VEGF in vascular smooth muscle cells[124] and macrophages.[125] These data suggest that the pro-angiogenic effects of PPAR-γ agonists could be one of the limitations to their clinical use. Therefore, the impact of new compounds developed for atherothrombosis therapy or as antiangiogenic therapy in other diseases should be tested on IPH risk before clinical use.

Due to the prominent role of oxidative stress in the intraplaque haemorrhage-dependent clinical expression of atherothrombosis in diabetic patients, vitamin E supplementation has been proposed in Hp2-2 diabetic patients for the prevention of atherothrombotic complications.[126] In the same way, anti-oxidant interventions have demonstrated their ability to prevent neovascularization in hypercholesterolaemic pigs.[127]

Conclusion

The newly established impact of intraplaque haemorrhages on the evolution of atherothrombotic plaques towards clinical expression

provides an innovative conceptual framework for future research and development in human atherothrombotic diseases. New biological challenges are to increase the understanding of how neo-angiogenesis is initiated in the early stages of human atheroma, why neo-vessels do not mature in the plaques, how intraplaque haemorrhages lead to plaque rupture and clinical expression, how cholesterol crystals impact plaque progression towards rupture, and how diabetes and other risk factors directly influence these phenomena These new concepts also reinforce the interest of exploiting human tissue and cell biobanks for research in cardiovascular diseases in general and in atherothrombosis in particular. They also underline the importance in tissue collection and diversification of sample preparation; i.e. chemical fixation for histology, direct freezing, preparation of conditioned media, smooth muscle and endothelial cell primary culture, leucocyte extraction, etc. These new paradigms will also impact translational research by promoting innovation in diagnostic tools (biomarkers, molecular imaging) and therapeutics in human atherothrombotic disease.

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