Biomarkers of Vascular Disease Linking Inflammation to Endothelial Activation

Part II

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Atherosclerosis is regarded as a dynamic and progressive disease arising from the combination of endothelial dysfunction and inflammation.1–6 This article is the second in a 2-part series examining emerging markers of inflammation and endothelial cell activation. The first article7 provided a brief overview of the link between inflammation, endothelial dysfunction, and atherosclerosis and began the examination of emerging inflammatory mediators. This second article continues with an exploration of more novel markers for cardiovascular disease.

Novel Inflammatory Mediators of Inflammation and Endothelial Cell Activation

Lectin-Like Oxidized Low-Density Lipoprotein (LDL) Receptor-1

The endothelial injury, activation, and dysfunction caused by oxidized LDL (oxLDL) in the pathogenesis of atherosclerosis are exerted via lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) activation.8,9 LOX-1, initially identified as the major receptor for oxLDL in endothelial cells, can also be expressed in macrophages and smooth muscle cells (SMCs).10–12 It is a type II membrane protein with a C-type lectin-like extracellular domain that can be cleaved, to release the soluble form of LOX-1, by an unknown protease.8 In addition to being the main receptor for oxLDL, LOX-1 has the ability to bind damaged or apoptotic cells, activated platelets, advanced glycation end products, and pathogenic organisms.13–15 Once bound, these ligands can be endocytosed or phagocytosed into the cell. Under physiological conditions, LOX-1 may play a role in host defense or serve to scavenge cellular debris.8 However, in pathological states, LOX-1 may be involved in binding proatherogenic materials, such as oxLDL, that activate the endothelium. With its ability to bind products that induce inflammation and endothelial activation, it is not surprising that elevated LOX-1 expression is observed in both initial and advanced atherosclerotic lesions.16,17

The stage for atherosclerosis is set once endothelial dysfunction occurs. LOX-1 may play a role in initiating and potentiating this crucial first step. Under conditions of hypercholesterolemia, hypertension, and diabetes, disease states that promote vascular injury, LOX-1 is highly expressed in blood vessels.9 Induction of LOX-1 expression is mediated by angiotensin II and endothelin-1, both antagonists of NO.18,19 With elevated levels of LOX-1 on the endothelium, increased amounts of oxLDL can be endocytosed, an activity that further enhances LOX-1 expression.20 OxLDL through LOX-1 also increases the expression of angiotensin-converting enzyme and reduces the intracellular concentration of NO.21,22 Thus, LOX-1 activity amplifies the extent of endothelial dysfunction. However, oxLDL uptake by LOX-1 also mediates endothelial cell apoptosis, potentially via nuclear factor (NF) κB activation.22 This may result in direct vascular denudation and injury that may trigger or enhance an existing inflammatory reaction.

In addition to setting the stage for atherosclerosis, LOX-1 activation contributes to the initiation and progression of atherogenesis. OxLDL, via upregulation of LOX-1, induces monocyte adhesion to the endothelium via enhanced expression of P-selectin, vascular cell adhesion molecule (VCAM), and intercellular adhesion molecule (ICAM).23 Receptor activation also results in monocyte chemotactic protein-1 expression, promoting monocyte migration into the intima.24 Enhanced expression of LOX-1 by macrophages within the plaque suggests that the receptor may be involved in oxLDL uptake and subsequent transformation into foam cells.8 LOX-1 upregulation in response to cytokines secreted by lymphocytes, such as tumor necrosis factor-α (TNF-α), further enhances lipid accumulation.25 Furthermore, it has recently been demonstrated that oxLDL through LOX-1 triggers the CD40/CD40L signaling pathway, which will perpetuate the inflammatory response.26 However, LOX-1 activation may also contribute directly to plaque instability.

OxLDL binding to LOX-1 induces increased production of intracellular reactive oxygen species (ROS), apoptosis of SMCs,
and modulation of matrix metalloproteinase (MMP) activity, toward conditions favoring compromise of the fibrous cap. 27–29 On plaque rupture, activated platelets may interact with the surrounding endothelium via LOX-1.30 In addition to thrombus formation, the activated platelets can further induce endothelial dysfunction in the surrounding tissue. Activated platelet–LOX-1 interaction promotes the release of endothelin-1 from endothelial cells and stimulates the generation of ROS that inactivate NO.15 Thus, LOX-1 signaling initiates a vicious circle of events that may potentially end in vascular occlusion and ischemic insult (Figure 1).

Protease-Activated Receptors (PARs)

PARs are a family of 7-transmembrane–domain, G-protein–coupled receptors that function to link tissue injury to appropriate cellular responses, such as inflammation and tissue repair, which may contribute to disease.31–33 PARs become activated by proteolytic cleavage of the amino terminus, which exposes a tethered peptide ligand that binds intermolecularly to the receptor. Currently, 4 PARs have been identified. PAR-1, PAR-3, and PAR-4 are activated by thrombin, whereas PAR-2 can become activated by trypsin, mast cell tryptase, and tissue factor.31 Within human atherosclerotic lesions and after vascular tissue injury, PAR expression is increased within these inflammatory environments.34 PARs are expressed by a variety of cell types in and around blood vessels, including endothelial cells, SMCs, and platelets.32 Within each of these cell types, PAR signaling can impact the initiation, progression, and complications of atherosclerosis (Figure 2).

Under the influence of the traditional cardiovascular risk factors, the endogenous defenses of the vascular endothelium begin to break down, resulting in endothelial dysfunction and injury. Injury to the endothelium not only elevates expression of PARs but also results in the initiation of tissue repair, in which thrombin plays a role.35 Endothelial cell PAR-1 activation promotes adhesivity toward monocytes via the induction of NFκB, which in turn promotes ICAM-1 expression.35 Similarly, PAR-4 activation, in which PAR-3 appears to serve as a cofactor, enhances leukocyte rolling, adherence, and recruitment, all early events in atherosclerosis.36,37 PAR-1 and PAR-2 activity induce discharge of Weibel-Palade bodies, endothelial storage granules containing the adhesion molecule P-selectin and von Willebrand factor, promoting both leukocyte and platelet adhesion.38 PAR activation is also linked to the secretion of interleukin (IL)–6, the cytokine that promotes C-reactive protein (CRP) synthesis, which itself triggers many of the steps in the inflammatory process.39 Recruitment of inflammatory cells perpetuates the proinflammatory activity of PARs given that IL-1 and TNF-α have been demonstrated to enhance expression of this receptor family.40 Overall, PAR activation appears to promote the inflammatory response within the intimal tissue, enhancing the initiation and progression of atherosclerotic plaques. However, the vascular effects of PARs appear to differ when compared with the endothelial dysfunction perpetuated by other inflammatory markers. Activation of PAR-1 and PAR-2 in human blood vessels has been demonstrated to produce NO and to result in vasodilation rather than the typical vasoconstrictor response secondary to NO degradation that occurs with CRP, LOX-1, and CD40/CD40L.41,42
The migration of vascular SMCs from the media to the intima and SMC proliferation are key processes in the progression of an atherosclerotic plaque. These activities have been linked, in part, to PAR activity in SMCs. Stimulation of PAR-2 induced SMC migration in vitro, migration that was inhibited using a PAR-2 blocking antibody. PAR-2 activity further serves as a mitogenic stimulus for coronary artery SMCs, suggesting the possibility of PAR-2 inhibitor use for the prevention of atherosclerosis or restenosis after balloon angioplasty. Also, fibrous cap formation is enhanced by PAR-1 activity, which promotes SMC collagen synthesis.

Finally, PAR-mediated activation of platelets may play an important role in plaque initiation and complication. As von Willebrand factor is released on PAR activity in endothelial cells, platelets are recruited locally to the lesion. Activation of platelets, mediated by PAR-1 or PAR-4 signaling, permits attachment to the atherosclerotic lesion, providing a reactive surface for the recruitment of monocytes and lymphocytes for plaque initiation. Platelet activation and aggregation after plaque rupture also arise from thrombin-mediated PAR activation that upregulates and activates the glycoprotein IIb/IIIa complex. The formation of these platelet thrombi, precipitated by unstable atherosclerotic plaques, may occlude the coronary arteries of the heart. Recent studies suggest that PAR-1 is the primary receptor that mediates the prothrombotic actions of thrombins in primates and that PAR-1 antagonists may have potential for the prevention of thrombus formation and vascular occlusion.

**Lipoprotein-Associated Phospholipase A₂ (Lp-PLA₂) and Secretory Phospholipase A₂ (sPLA₂)**

The potential for Lp-PLA₂, also known as the platelet-activating factor acetylhydrolase, to serve as a new risk factor for coronary artery disease emerged from studies examining the levels of plasma Lp-PLA₂ in hypercholesterolemic patients. Results from the West of Scotland Coronary Prevention Study Group suggest that plasma levels of Lp-PLA₂ appear to be a strong risk factor for a coronary event, a finding that potentially implicates Lp-PLA₂ in hypercholesterolemia. Levels of Lp-PLA₂ have also been reported to be significantly elevated in patients with angiographically proven coronary artery disease and appear to serve as an independent predictor of disease status in this population. However, a prospective evaluation of Lp-PLA₂ levels as a
risk of future cardiovascular events in apparently healthy middle-aged women suggested that it is not a strong predictor. Furthermore, some experimental evidence suggests that Lp-PLA₂ may actually function in an antioxidant, anti-inflammatory, and antiatherogenic capacity (reviewed in Tselepis and Chapman). Our discussion will focus on the evidence suggesting a proinflammatory and proatherogenic role for Lp-PLA₂.

Lp-PLA₂ is a monomeric enzyme that catalyzes the hydrolysis of the sn-2 ester bond, preferentially when short acyl groups are at the sn-2 position, of oxidized phospholipids. In the plasma, 80% of Lp-PLA₂ is found bound to LDL, primarily small dense LDL, and ~20% is associated with high-density lipoprotein. Combined in situ hybridization and immunocytometry studies detected Lp-PLA₂ mRNA and protein expression by macrophages in both rabbit and human atherosclerotic lesions, suggesting its activity within the plaque. This enzyme’s activity generates 2 bioactive products, namely lysophosphatidylcholine (lysoPC) and non-esterified fatty acid moieties, which may potentiate atherosclerosis. LysoPC is a potent chemotactic factor for monocytes and induces the expression of mononuclear leukocyte adhesion molecules in endothelial cells. Thus, it may enhance lesion initiation. Once lymphocytes are recruited into the developing plaque, lysoPC may complex with peptides and serve as antigen to stimulate T cells. On T-cell activation, the immune reactions within the vessel wall are not only sustained but enhanced. As the plaque progresses, lysoPC may promote vascular SMC proliferation by activating DNA synthesis. However, at higher concentrations, potentially after prolonged oxLDL synthesis and Lp-PLA₂ activity, lysoPC induces a combination of apoptotic and nonapoptotic death in SMCs. As a result, Lp-PLA₂ activity may eventually lead to plaque destabilization, increasing the possibility of rupture and thrombosis. Furthermore, Lp-PLA₂ activity may increase oxLDL toxicity toward macrophages, leading to foam cell death and the release of several proatherogenic compounds. Inhibition of Lp-PLA₂ by a selective blocker did not prevent LDL oxidation, but it did inhibit the rise in lysoPC, which appeared to diminish the cytotoxicity and apoptosis-inducing effects of oxLDL on macrophages. Thus, inhibiting Lp-PLA₂ may present a new therapeutic target limiting atherosclerosis (Figure 2).

Type II sPLA₂, an acute-phase reactant, may also represent a new independent risk factor for coronary artery disease. Detected in human atherosclerotic plaques using immunohistochemical staining, but not in normal arteries without signs of ongoing inflammatory reactions, its biological roles may include antibiotic function, contribution to the removal of injured or apoptotic cells, and enhancing the clearance of cholesterol from the bloodstream. However, within plaques, it can play a proatherogenic role. Like Lp-PLA₂, sPLA₂ is able to catalyze the sn-2 ester bonds of glycerophospholipids resulting in the generation of lysophospholipids, such as lysoPC, which may trigger inflammatory cellular processes as discussed above. The cellular components of the plaque, SMCs, endothelial cells, mast cells, and macrophages, possess the ability to synthesize sPLA₂. Secretion of sPLA₂ is induced by proinflammatory compounds within the atherosclerotic environment such as IL-1, IL-6, TNF-α, interferon-γ, and oxLDL, enhancing plaque formation.

Plaque formation is further enhanced via the role of sPLA₂ in increasing intracellular lipid accumulation within macrophages leading to foam-cell formation. sPLA₂ activity has been implicated in promoting monocyte chemoattractant protein-1 secretion by monocytes as well as inducing the surface display of functional CD40 on monocytes. Thus, sPLA₂ may serve as a link between the lipid and the inflammation hypothesis of atherosclerosis. This potential link is strengthened by results from studies in transgenic mice that express human group II sPLA₂. Compared with nontransgenic littermates, the transgenic mice exhibited significant atherosclerotic lesions when maintained on a low-fat diet possibly as a result of the elevated levels of biologically active oxidized phospholipids in these mice, which result in an increased ability to stimulate monocyte–endothelial cell interactions. Furthermore, circulating levels of sPLA₂, increased in various chronic inflammatory diseases, have been demonstrated to independently predict clinical coronary events in patients with unstable angina and documented coronary artery disease.

Matrix Metalloproteinase-9

MMPs are a family of zinc-containing enzymes with proteolytic activity against extracellular matrix components such as elastin, proteoglycans, and collagen. More than 20 mammalian MMPs have been cloned and identified, all of which are inhibited by specific tissue inhibitors of metalloproteinases (TIMPs). MMPs play an important role in vascular remodeling, which permits changes in size and composition of adult blood vessels, allowing for adaptation and repair. However, with excessive MMP expression and activity, inappropriate cardiovascular remodeling may occur, resulting in cardiac dysfunction, aneurysmal dilatation, restenosis, and atherosclerosis.

MMPs have been identified in human atherosclerotic plaques, especially at the shoulder region, and in areas of foam-cell accumulation. Among these MMPs is MMP-9 (gelatinase B, 92-kDa type IV collagenase); MMP-9 is capable of degrading gelsatins, fragments of collagens degraded by collagenases, and type IV collagen, which forms basement membranes. The role of MMP-9 in cardiovascular disease is supported by genetic studies that showed that variations in the MMP-9 gene were related to the presence and severity of atherosclerosis. Circulating MMP-9 levels are increased in type 2 diabetic patients with coronary artery disease, and elevated serum MMP-9 concentrations, associated with decreased TIMP levels, have been linked to premature coronary atherosclerosis. Recently, plasma MMP-9 concentration was identified as a predictor of cardiovascular mortality in patients with coronary artery disease. Furthermore, the plaque-stabilizing effects of statins may be related to their ability to inhibit MMP-9 secretion.

MMP-9 appears to participate in several stages of atherosclerosis. Matrix degradation is a prerequisite for monocyte recruitment because cell transmigration across the endothelium requires disruption of the basement membrane. Expression of MMP-9 is enhanced on femoral arterial injury and was
shown to increase on monocytic cell–endothelial cell interaction in vitro.80,81 Inside the vessel wall, macrophage foam cells and SMCs secrete MMP-9 in response to oxLDL, ROS, TNF-α, and IL-1.73 MMP-9 activity causes the degradation of the basement membrane surrounding each SMC, permitting SMC migration and fibrous cap formation.82 However, excessive proteolytic activity by MMPs results in destabilization of the plaque83 (Figure 3).

The plasma levels of MMP-9 are significantly increased in the coronary circulation in patients with acute myocardial infarction and unstable angina.84 Overexpression of MMP-9 promotes intravascular thrombus formation by degrading the fibrous cap, and coagulation is further enhanced by MMP-9–mediated cleavage of tissue factor pathway inhibitor.85 Finally, MMP-9 has also been demonstrated to mediate the release of kit ligand, promoting the mobilization of stem cells from the bone marrow.86 This may prove to serve as another mechanism by which MMP-9 promotes atherosclerosis and restenosis, as indicated by the recent finding that stem cells differentiate into vascular SMCs that contribute to pathological arterial remodeling.87

**Endothelial Progenitor Cells (EPCs)**

EPCs are bone marrow–derived stem cells that have the ability to differentiate into functional endothelial cells (reviewed in Szmitko et al88). EPCs circulate in the blood and appear to home preferentially to sites of vascular or tissue injury, contributing significantly to both reendothelialization and neovascularization.89,90 Thus, EPCs may play an important role in maintaining an intact and functional endothelium that resists atherosclerosis. However, if EPC mobilization from the bone marrow and recruitment to sites of vascular injury are impaired, vascular homeostasis may be deviated toward endothelial dysfunction and susceptibility to atherogenesis. In fact, a recent study suggested that in healthy men, levels of circulating EPCs may be a surrogate biological marker for vascular function and cumulative cardiovascular risk.91 The authors of that study propose that EPCs maintain endothelial function in mature blood vessels and that the failure of this function may lead to abnormal vasoreactivity. The detrimental effect on EPC number may arise from impaired mobilization or a depletion of EPC supply due to continuous endothelial damage and repair.91 Furthermore, the number and migratory activity of circulating EPCs is decreased in patients with risk factors for coronary artery disease, suggesting that a lack of EPCs may contribute to impaired vascularization within these patients.92 Also, statin therapy, shown to have favorable effects in reducing morbidity and mortality from coronary artery disease, can enhance the mobilization of EPCs from the bone marrow, reduce EPC senescence, increase EPC proliferation, and thus lead to an increased number of EPCs within the circulation.93–95 Therefore, EPC dysfunction may in turn result in endothelial dysfunction. Recent work by our group has shown that CRP, a proatherogenic, inflammatory mediator, can have a detrimental effect on EPC differentiation and survival, an observation that strengthens the link between inflammation and cardiovascular disease.96 However, a reduction in circulating...
EPCs may go beyond simply setting the stage for atherosclerosis. A reduction in the number of circulating EPCs may also result in the impaired reendothelialization of eroded plaques and thus lead to a greater propensity for thrombosis and vascular occlusion (Figure 4). There is still a great deal to learn about EPC mobilization and function within the context of inflammation and atherosclerosis. The information gained from future studies may help to determine how best to modify and to use the body’s own regenerative potential to combat endothelial dysfunction and to prevent ensuing cardiovascular disease.

**Conclusions**

Atherosclerosis is no longer considered a pure lipid disorder. It has become increasingly clear that inflammation is at the root of atherosclerosis and its complications. In addition to playing a causal role in lesion formation, inflammation can yield predictive and prognostic information of considerable clinical utility. A number of mechanisms and mediators of inflammation have been identified, of which high-sensitivity CRP has emerged as one of the most important. In addition to serving as biomarkers of atherosclerotic events, inflammatory mediators directly participate in lesion formation, propagation, and eventual rupture and in this fashion may represent a powerful tool to assess endothelial cell activation. Clearly, understanding the mechanisms and mediators of endothelial dysregulation and inflammation may yield new targets to predict, prevent, and treat cardiovascular disease.

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**References**


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