

Proinflammatory circulating molecules in peripheral arterial disease (Review)

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Abstract. Peripheral arterial disease (PAD) affects about 5% of the elderly population in the western world. It has been reported that PAD is associated with elevated plasma levels of the inflammatory markers. The goal of this review is to describe which proinflammatory molecules may play a role in PAD development, C-reactive protein, fibrinogen, pro-inflammatory cytokines, adhesion molecules and matrix metalloproteinases have been reported to be involved. High serum levels of both C-reactive protein and fibrinogen have been shown to be significantly associated with increasing severity of PAD. Among cytokines, IL-6 is one of the most studied in PAD. IL-6 is well recognized for its role in the acute phase inflammatory response which is characterized by production of a variety of hepatic proteins termed acute phase proteins. It has been shown that increased serum concentrations of several markers of the acute response, including IL-6, are elevated in patients with type 2 diabetes. These patients have a two-fold risk of PAD compared to those without type 2 diabetes. The G(-174)C IL-6 polymorphism has been suggested to influence IL-6 release, and its possible influence on PAD development among individuals with type 2 diabetes is discussed in this review. Further study of these molecules is justified as they appear to be involved in the development of PAD.

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1. Introduction

The term peripheral arterial disease (PAD) is widely used to refer to chronic arterial disease of the legs of atherosclerotic origin. Atherosclerosis is by far the most common cause (>90%) of arterial problems in the leg (1). PAD affects about 5% of the elderly population over 55 years in the western world (2). Several studies have demonstrated that inflammation is also involved in the development of PAD (3-9).

Previous studies have demonstrated that PAD is closely associated with elevated plasma levels of the inflammatory markers C-reactive protein (CRP) and fibrinogen (10,11). Moreover, other studies have focused on the association between such proinflammatory markers and PAD in patients with diabetes and the arterial consequences of diabetes, such as PAD. Accordingly, diabetes shows a high prevalence in the general population and is a crucial risk factor for cardiac and arterial disease (12-15).

The pivotal and crucial role played by inflammation in the pathophysiology of PAD has received much attention. Based on our current knowledge, we propose that inflammatory mediators may be involved in PAD development (Fig. 1). However, inflammation may also be associated with hypercoagulability (16), and to date we know that both raised levels of markers for inflammation and a high pro-coagulative status have been associated with increased risk for cardiovascular disease (CVD) (17). It has been recognized that inflammation may contribute to all stages of the atherosclerotic process (18), and considerable evidence indicated that the activated haemostatic system also plays an important role in the pathophysiology of atherosclerotic vascular disease. Pathological studies of post-mortem arteries and samples obtained during reconstructive vascular surgery related the progression of atherosclerosis to the extent of fibrin deposition and its degradation products in the arterial wall (19-21). It has been suggested that serum concentrations of inflammatory proteins, such as CRP, are associated with cardiovascular risk because they reflect the severity of the

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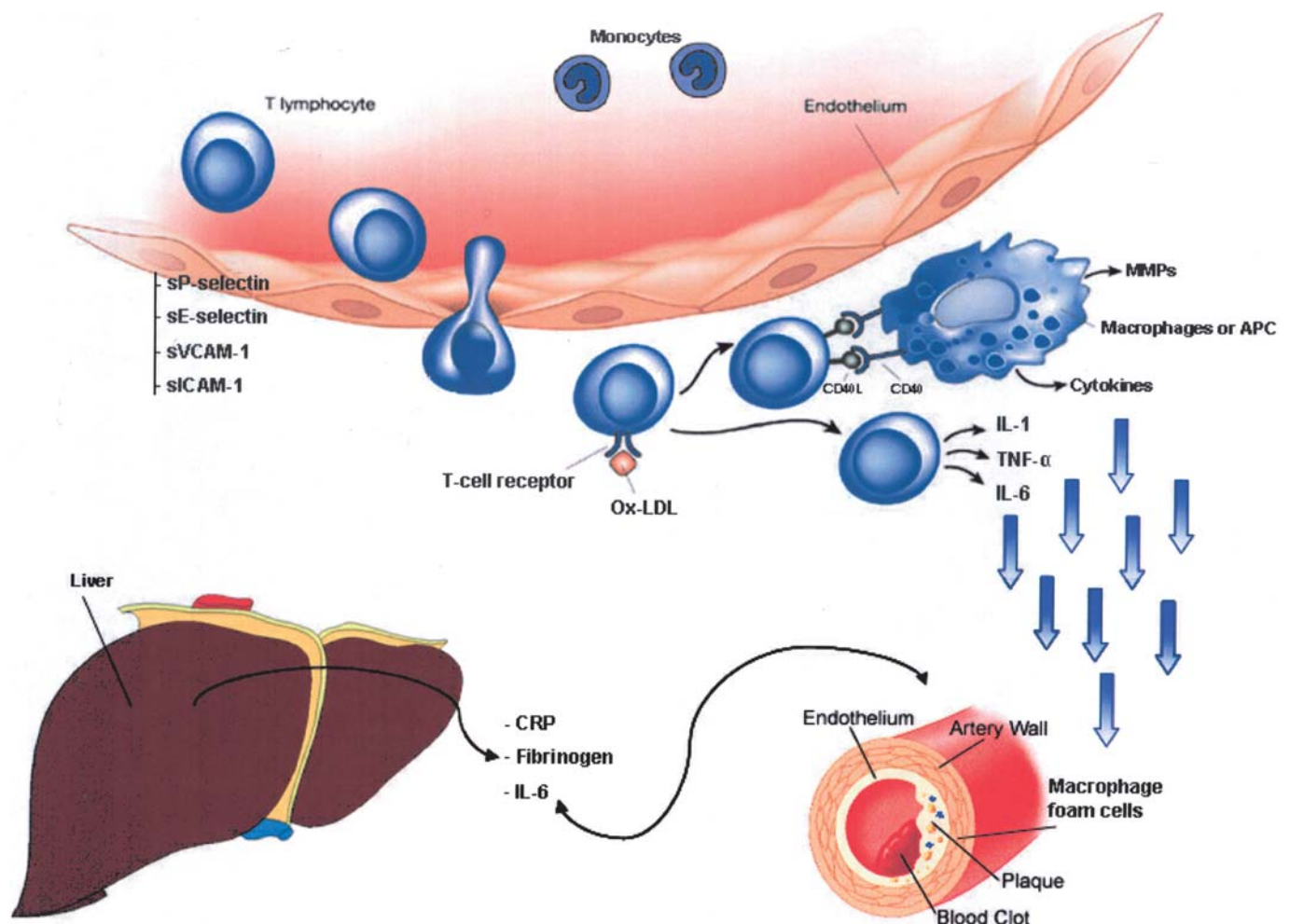


Figure 1. Inflammation process in peripheral arterial disease. This figure schematizes the migration of leukocytes through the endothelium. Once resident in the intima, the monocyte acquires characteristics of the tissue macrophage, the T cells activated by antigens such as oxidized low-density lipoprotein (Ox-LDL) produce cytokines that can influence the behaviour of other cells such as macrophages. Of note, CD40 binding to its ligand, on macrophages, may induce the expression of matrix metalloproteinases (MMPs) and proinflammatory cytokines. Crosstalk of these mediators, including those produced by liver, may contribute to inflammatory mechanisms in PAD.

atherosclerosis (22). A characteristic feature of the acute phase response is increased hepatic synthesis of a heterogeneous group of plasma proteins termed acute-phase proteins, which includes CRP, serum amyloid A (SAA), haptoglobin, and α 1-acid glycoprotein (23).

Measurements of serum acute-phase parameters allow a sensitive quantification of the vascular inflammatory process. The vascular inflammatory process is suggested to stimulate both vascular smooth muscle cell proliferation and late neo-intimal growth. Vascular smooth muscle cell proliferation and hypertrophic neo-intimal formation at the treated segment frequently lead to stenosis of artery and also to restenosis of arterial graft (24,25). Since the acute-phase response may induce damage of the vascular endothelium that is a crucial step towards the atherosclerotic plaque, plasma levels of inflammatory mediators may simply represent the extent of the atherosclerotic process. Alternatively, however, increased local or systemic inflammation due to chronic infection, chronic inflammatory disease, smoking, obesity, or impaired glucose tolerance (26-29) may precede the progression of atherosclerosis. Increased plasma levels of inflammation markers have been identified in patients with a risk of atherosclerotic

diseases in many epidemiological studies. Thus, CRP (30), fibrinogen (31), D-dimer (32,33) and SAA (34,35) have been found to be elevated in patients with an increased risk of developing arterial consequences of atherosclerosis. It now appears that an imbalance of haemostatic factors may be involved in PAD. High blood levels of fibrinogen and homocysteine play a part and a strong association was found with PAD (1).

Elevated plasma levels of fibrinogen have been shown to predict the future risk of cardiac death and myocardial infarction in stable claudicants, and high levels were found in patients with more severe peripheral atherosclerosis (17,36,37). Increased levels of haemostatic factors (e.g. D-dimer, tissue plasminogen activator antigen, plasminogen activator inhibitor, and prothrombin fragment 1, 2) and inflammatory markers (e.g. CRP, fibrinogen, and SAA) may be associated with functional impairment because they are sensitive measures of the burden of the atherosclerotic process of arteries in the lower leg (18,21,22,31,32,38). In addition, those haemostatic components were increased in patients with PAD compared with patients without PAD (33,38,39). In prospective epidemiological studies of atherosclerotic patients as well as healthy subjects, baseline

abnormalities in several coagulation and fibrinolytic variables, including plasma fibrinogen, fibrin degradation products, coagulation factor VII and factor VIII, and tissue-type plasminogen activator have been associated with increased risk of acute thrombotic events (21,36,37,40-43). In addition, increased fibrin D-dimer reflecting an overall increased coagulation has been demonstrated in patients with PAD (44).

2. C-reactive protein

CRP is a sensitive acute-phase reactant (45) although its function is not well defined. It binds to damaged tissue and nuclear antigens in a calcium-dependent manner, activating complement and generating proinflammatory cytokines. It is thus believed to have a proinflammatory role (46). Several prospective studies have found CRP to be a strong and independent predictor of major cardiovascular adverse events in apparently healthy men and women (47-49). It is probable that CRP itself plays a significant role in the progression of atherosclerosis (49).

In contrast to the studies involving patients with coronary artery disease (CAD) there are only a limited number of studies which have assessed CRP levels in patients with PAD. Minor elevations of CRP have been shown to be predictive of cardiovascular events in patients with coronary heart disease (50). It is now believed that CRP is not merely a marker of low-grade chronic systemic inflammation but may be actively involved in atherosclerosis as it can amplify the inflammatory response through complement activation, tissue damage and activation of endothelial cells (51). Ridker and colleagues have shown CRP levels to be a strong predictor of future cardiovascular events based on a study of 28,000 women (49). McDermott and colleagues, have shown that CRP was not associated with ankle-brachial pressure index in patients without a history of cardiac or cerebrovascular disease (33).

Many studies indicated that blood coagulation cascade is activated in patients with PAD (47). The direct effect of fibrinogen on atherosclerosis is through several processes, such as: a) its effect on clot structure, since increased plasma fibrinogen levels result in fibrin matrices with a tight structure that are difficult to lyse; and b) fibrinogen induces chemotaxis of smooth muscle cells and monocytes/macrophages (52).

3. Fibrinogen

Blood viscosity and its major determinants (plasma viscosity, haematocrit and fibrinogen) have been shown to be significantly associated with increasing severity of PAD, and with early evidence of atherosclerosis (measured by carotid intima-media thickness) (53), and studies have analyzed the role of coagulative factors in diabetics with PAD (54-59). Fibrinogen has been implicated in the increased risk of PAD in diabetic populations (54-61).

4. Cytokines

Cytokines, such as interleukin (IL)-6 and tumor necrosis factor (TNF)- α , are soluble polypeptides acting as important humoral regulators in immunoregulation, hematopoiesis, and the

inflammatory cascade (62,63). CRP is an end-product of inflammation whose synthesis by the liver is stimulated by cytokines (64). Circulating cytokine receptors may provide additional information in chronic inflammatory processes because they generally have a longer half-life than the cytokines themselves and, therefore, show more constant levels over time (65,66).

Interleukin-6. Among cytokines, IL-6 is one of the most studied in PAD. IL-6 contributes to a myriad of physiologic and pathophysiologic processes (67). Because of the large scope of its effects, the cellular and molecular biology of IL-6 has been explored by a variety of investigators representing a great number of basic biological and medical fields. IL-6 is well recognized for its role in the acute-phase inflammatory response which is characterized by production of a variety of hepatic proteins termed acute-phase proteins (e.g., C-reactive protein, serum amyloid A, fibrinogen, complement, α 1-antitrypsin) (68).

G(-174)C IL-6 polymorphism. It has been shown that increased serum concentrations of several markers of the acute response, including IL-6, are elevated in patients with type 2 diabetes (69,70). Individuals with type 2 diabetes have a two-fold risk of PAD compared to those without type 2 diabetes (71). The G(-174)C IL-6 polymorphism has been suggested to influence IL-6 release (72). Recently our group determined whether the G(-174)C IL-6 polymorphism may influence development of PAD among individuals with type 2 diabetes. This possibility was investigated by comparing the distribution of G(-174)C genotypes among subjects with type 2 diabetes and PAD to that of subjects with type 2 diabetes without PAD. The data reported by Libra *et al* (73) showed that the GG genotype was more frequently present among diabetics with PAD than those without PAD. This finding suggests that individuals with type 2 diabetes and the GG genotype may develop PAD more often than those with GC or CC genotypes.

Previous studies comparing plasma IL-6 levels to G(-174)C genotype have yielded conflicting results. Some investigators detected increased plasma IL-6 levels among those with the GG genotype while others observed increased plasma IL-6 levels among those with the CC genotype (72,74-76). Plasma IL-6 levels have also been reported to be independent of the G(-174)C genotype (77,78). Libra *et al* indicate that both diabetics with and without PAD with the GG genotype have higher mean plasma IL-6 levels than those with GC or CC genotypes. An analysis of plasma levels of IL-6, fibrinogen, and CRP among abdominal aortic aneurysm patients reveals that only IL-6 plasma levels vary among patients with different G(-174)C genotypes (79). In contrast, the GG genotype is associated with increased mean plasma levels of IL-6 as well as fibrinogen and CRP in the study conducted by Libra *et al*. Therefore, it is possible that the GG genotype facilitates increased IL-6 release among individuals with type 2 diabetes that then causes increased release of fibrinogen and CRP. This possibility is further supported by the observation that plasma levels of IL-6, fibrinogen, and CRP among PAD-positive subjects were each correlated with one another (73). Thus, the GG genotype promotes development of PAD among individuals with type 2 diabetes by inducing increased release

of IL-6. Elevated release of IL-6 into the bloodstream in those with the GG genotype causes increased release of fibrinogen and CRP. These findings are consistent with previous studies that have documented increased incidence of CVD among individuals with the GG genotype compared to those with the CC genotype (75,76,80,81). However, other studies have associated CVD with the CC genotype instead of the GG genotype (79,82-84). The conflicting results that have been reported suggest that there may be a complex relationship between G(-174)C genotype and development of CVD. The relationship between G(-174)C genotype and PAD development may differ among individuals with different clinical characteristics.

5. Adhesion molecules and matrix metalloproteinases

The role of proinflammatory circulating molecules in vascular disease is clear (85-87); activated leukocytes emigrate, adhere to the endothelial wall and migrate through the arterial wall, resulting in the transfer of macrophages rich in oxidized lipoproteins that trigger the onset of atherosclerotic plaque formation (88-91). In contrast, few studies have been conducted on the response of adhesion molecules in patients with PAD (92-100). Our previous studies have shown higher levels of soluble inflammatory mediators and adhesion molecules such as E-selectin, L-selectin, P-selectin, VCAM-1 and ICAM-1 in patients with PAD than in controls. Blood samples were also analyzed at rest and after maximal treadmill test (101). The molecules analyzed were higher both at rest in PAD than in controls, these increased in both patients and controls after a maximal treadmill test but a significant difference was found comparing the after-test serum levels between PAD and controls. This suggests a close relationship between physical stress and activation of white blood cells that in turn are able to release the adhesion molecules into the bloodstream. Therefore, it is suggested that increased serum concentrations of both cytokines and adhesion molecules can determine further changes in leukocytes (rolling, adhesion) and lead to endothelial damage. Atherosclerosis is also characterized by degeneration of the extracellular matrix (ECM) proteins. Degradation occurs as a consequence of complex interactions between genetic factors, inflammatory cytokines, matrix metalloproteinases (MMPs), tissue inhibitors of MMPs (TIMPs), and others. The resulting phenotype is dissolution and fragmentation of collagen and elastin, leading to expansion of the vessel wall. MMPs are a family of Zn^{2+} -dependent enzymes that catalyze the proteolysis of many connective tissue proteins of the ECM such as collagen, gelatin, fibronectin, laminin, elastin, and proteoglycans (102,103). Other MMP substrates are not components of the ECM. MMPs have been implicated in the degradation of myelin basic protein (104), and interleukin-1 β (105), as well as proteolytic processing of tumor necrosis factor- α (106). MMPs are secreted as proenzymes by many cell types (107), including leukocytes, macrophages, astrocytes, neurons, and microglia, and are widely distributed in tissues and biological fluids such as blood and urine (108). They are involved in many physiological processes, including tissue remodeling during development and platelet aggregation (109). MMPs also have role in pathophysiological processes such as inflammation, tissue

repair, myocardial injury, vascular diseases, tumor invasion, and metastasis (110-112). Activity of MMPs is negatively regulated by tissue inhibitors of matrix metalloproteinases (TIMPs) (79). The *in vivo* balance between MMPs and TIMPs dictates the level of MMP activity (113). Deterioration of MMP regulation contributes to the development of arterial lesions, in part, by facilitating monocyte invasion (114).

Physiologically, extracellular matrix proteins maintain the integrity of the vessel wall. In fact, degradation of these proteins is minimal because they are expressed at low levels and in inactive forms (115). The activation of MMPs requires the removal of an amino-terminal sequence by plasmin and the plasmin-generating enzymes tissue-type plasminogen activator and urokinase-type plasminogen activator, which are minimally expressed in the normal vessel wall. In addition, activated inflammatory cells, which are the main sources of collagenolytic and elastinolytic proteinases, are also absent. Furthermore, expressed TIMPs neutralize the proteolytic activities of MMPs. Collectively, these processes maintain the integrity of the extracellular matrix proteins and the vessel wall.

Gelatin zymography studies have shown that MMPs, especially MMP-2 (72-kDa gelatinase A) (116,117) and MMP-9 (92-kDa gelatinase B) (118), are involved in remodeling processes associated with atherogenesis (119,120). MMPs are synthesized in atheromatous plaques (121) and elevated levels are present in rupture-prone shoulder regions of arterial vessels (122). Increased MMP activity has also been correlated with CVD (123,124).

Several studies have investigated the potential involvement of MMPs in atherosclerosis. For example, expression of MMP-2 was shown to be increased within atherosclerotic plaques (125,126). Other studies have implied that expression and activity of MMPs may be increased among type 2 diabetes (112,127-129). Death *et al* showed that exposure to a high concentration of glucose induced increased expression and activity of MMP-1 and MMP-2 in endothelial cells and MMP-9 in macrophages (112). Plasma levels of MMP-9 and other markers of inflammation were decreased in type 2 diabetics by treatment with rosiglitazone, recently introduced for the management of diabetes (127,128). Portik-Dobos *et al* have reported that MMP-9 levels are lower in blood vessels isolated from type 2 diabetics than from normal individuals (129). Data from our studies have indicated that MMP-9 is released from blood vessels into the bloodstream in patients with type 2 diabetes and PAD to a greater extent than in healthy individuals, which may contribute to increased chronic local inflammation of blood vessels among type 2 diabetics. Additionally, the endothelium in blood vessels from diabetics may activate MMP-producing cells in the circulation (130).

6. Conclusion

This article describes the involvement of pro-inflammatory molecules in the pathophysiology of PAD both for atherosclerotic and diabetic patients. More inflammatory markers were found in the serum of patients with PAD and we speculate that these markers could be considered as relevant in monitoring the course of the disease. Accordingly, we must consider that activated white blood cells and endothelial

dysfunction, and a lack of fibrinolytic activity of endothelium membrane and consequently presence of coagulative status can play a crucial role in worsening the blood supply towards a critical reduction, especially in microcirculation, and overall these factors lead to critical limb ischemia as a severe clinical haemodynamic situation in the lower limbs. Additionally, proving a pathogenic role of cytokines could aid the development of novel therapeutic approaches for the prevention and also for the management of PAD by negating the action of endogenous cytokines. This is noteworthy considering that all vasoactive drugs usually given for treatment of PAD were judged unable to fulfil all the targets of the therapy (131). However, the role of the MMP family in PAD development has been studied and, therefore, we should consider how this knowledge may also influence the treatment of PAD.

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