Calcification and C-reactive protein in atherosclerosis: effects of calcium blocking and cholesterol lowering therapy
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C-reactive protein, risk factor versus risk marker

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Abstract

Purpose of this review

C-reactive protein (CRP) is consistently associated with cardiovascular disease (CVD) in prospective and cross-sectional clinical and epidemiological studies. Inflammation is an important mechanism in CVD, and the plasma level of CRP is considered to reflect the inflammatory condition of the patient and/or the vessel wall. In addition, there are also a number of indications for a causal role of CRP in CVD.

Recent findings

A number of new publications show potential causal effects of CRP on CVD, and evidence from human-CRP transgenic animals also indicates a causal contribution of CRP to CVD. On the other hand, a new large prospective study and an updated meta-analysis indicate that the contribution of CRP to CVD is less impressive than reported earlier (OR=1.58, 95% confidence interval, 1.48 to 1.68).

Summary

We review here the most recent evidence about mechanisms by which CRP is involved as a causal factor in the precipitation of CVD. Evidence for such a role is accumulating.

Keywords: C-reactive protein, cardiovascular disease, atherosclerosis, thrombosis, inflammation
Introduction

Inflammation is a major mechanism in cardiovascular disease (CVD)\(^1,2\), and it has been studied extensively whether the plasma concentrations of circulating inflammatory variables are predictors of CVD. Indeed, elevated levels of several inflammatory factors (e.g. C-reactive protein, fibrinogen, interleukin-6) consistently predict the risk of CVD.\(^3,4\)**

The mechanism underlying the relationship between inflammatory variables, such as CRP, and CVD is complex and has not yet been fully elucidated. CRP is an acute phase protein and increased levels reflect inflammation, in this context the inflammatory condition of the vascular wall. It is now generally accepted that this reflection of the inflammatory state explains a great part of the association between CRP and CVD. But there are strong indications that CRP also contributes directly to the progression of atherosclerosis and the precipitation of cardiovascular events.

CRP levels as a combination of both risk marker and causal factor fits within the response-to-injury hypothesis of atherosclerosis that has been put forward by Russell Ross and which states that the protective, inflammatory response can be followed by the formation of a fibroproliferative response, which begins as a protective mechanism but that with time and continuing insult may become excessive\(^5\). In its excess, both inflammation and fibrous connective tissue proliferation become, in themselves, the disease process. This would be the essence of the process of atherogenesis. In the light of this hypothesis of Russell Ross, chronically elevated levels of inflammatory factors indicate that the individual is not capable of regulating his/her inflammatory process.

A first indication that CRP is more than a risk marker was the observation that, of the inflammatory markers studied (such as P-selectin, interleukin-6, interleukin-1, tumour necrosis factor-\(\alpha\), soluble intercellular adhesion molecule-1, fibrinogen), CRP emerged as the most powerful inflammatory predictor of future cardiovascular risk\(^6,7\). In addition, several functional characteristics of CRP that are also associated with initiation and progression of CVD are already known for a long time, such as activation of the classical route of complement activation\(^8\). Recently, a number of biological effects of CRP have been reported that may be of relevance for CVD. In this concise review we briefly summarize the role of CRP as CVD marker and of the recent data on biological effects of CRP.

**CRP as marker of cardiovascular risk**

CRP levels have consistently been associated with CVD in many clinical and population studies, with cross-sectional and prospective designs. Recently, Danesh and colleagues
published an updated meta-analysis that shows that the relationship between CRP levels and risk is lower than reported previously (new estimate of Odds Ratio = 1.58; 95% confidence interval, 1.48 to 1.68)**. The authors explain this lower estimate of the Odds Ratio by preferential publication of positive results in earlier studies. With an Odds Ratio of 1.58, it remains to be seen whether the clinical value of CRP as a predictor of risk of CVD is important enough to add the CRP measurement to the routine package, as recommended by the American Heart Association9.

Several studies have evaluated whether adding CRP to a risk score improves the risk prediction, but the conclusions are not consistent10. Whether the CRP test should be used to assign statin treatment is being investigated in a recently started, large-scale, randomized clinical trial (JUPITER). This trial tests whether rosuvastatin therapy will reduce CVD incidence in subjects with elevated plasma CRP levels who do not fulfill the standard criteria for lipid-lowering treatment1. The study is based on the hypothesis that the pleiotropic effects of statins, such as lowering of CRP levels, have an important contribution to the benefits of statin treatment.

What is remarkable, and supported by several recently published articles, is that CRP is associated with a wide number of outcome variables. Among those are associations between CRP and stroke, severity of atherosclerosis, outcome after percutaneous coronary intervention11,12. These associations have been obtained from various patient groups, such as patients with renal failure13, diabetes14, hypertension15, old16 and young patients. Recently, it was reported that in patients with unstable angina and elevated C-reactive protein levels, the carotid artery plaques are less stable, which may result in rapid plaque growth and atherosclerotic plaque instability.17**

In humans, it is impossible to obtain evidence for a direct contribution of CRP to atherosclerosis development since one cannot distinguish between the role of CRP levels as a reflection of the underlying inflammation of the vascular wall and the direct, causal role of CRP to CVD. In mice, CRP is not a strong acute phase protein and that makes the mouse a very interesting model to study the contribution of CRP to atherosclerosis, although it has to be considered that extrapolation from mouse-studies to humans has to be done with caution. Recently, Paul and colleagues18* cross-bred transgenic mice that express the human CRP gene with the (apo)E−/− mice that can develop atherosclerosis. They observed that aortic atherosclerotic lesions in 29-week-old male CRPtg10/apoE−/− mice were 48% larger in turpentine-treated mice and 34% larger in untreated CRPtg10/apoE−/− mice than in CRPtg00/apoE−/− littermates. It has to be realized, however, that in this study the CRP levels in the CRP mice were above 100 mg/l, whereas normal levels in human subjects are ± 2 mg/l.
Mechanisms of causal CRP involvement in CVD, focusing on atherosclerosis

Inflammatory mechanisms play a central role in all phases of atherosclerosis, from initial recruitment of circulating leukocytes to the arterial wall to the rupture of unstable plaques resulting in clinical manifestations of the disease. CRP may be causally involved in each of these stages by influencing processes such as endothelial dysfunction, monocyte recruitment and activation, lipid-related effects, complement activation, angiogenesis and apoptosis, and thrombosis. The role of CRP in these processes will be described in more detail below.

Endothelial dysfunction

Endothelial dysfunction is one of the early abnormalities in atherosclerosis, characterized by upregulation of adhesion molecules on the endothelial surface, which allows adhesion and subsequent transmigration of monocytes into the vessel wall. CRP can induce the expression of adhesion molecules such as intercellular adhesion molecule (ICAM-1), vascular cell adhesion molecule (VCAM) and E-selectin, in human endothelial cells\(^ {19}\). The CRP-induced increase in expression of adhesion molecules resulted in elevated adhesion of monocytoid U937 cells to endothelial cells in vitro\(^ {20}\). These findings were confirmed by others\(^ {21,22}\) who additionally showed that CRP induced monocyte chemoattractant chemokine-1 (MCP-1) production. The effects are partly mediated via the production of endothelin-1, a potent endothelium-derived vasoactive factor, and by the production of the inflammatory cytokines interleukin-6 (IL-6) and IL-8. As to the effects of CRP on MCP-1 expression, aortic endothelial cells seem to be unresponsive whereas venous endothelial cells show increased MCP-1 expression\(^ {21,23,20}\). Atherosclerosis mainly develops in the arteries and the clinical significance of the effect of CRP on venous cells is unclear.

CRP reduces the expression and bioactivity of endothelial nitric oxide synthase (eNOS or NOS\(_3\)) in human aortic endothelial cells (HEACs)\(^ {24,25}\). Moreover, CRP reduces prostacyclin activase activity resulting in a decreased prostacyclin release\(^ {24,25}\). Less eNOS activity reduces the bioavailability of nitric oxide, which results in inhibition of vasodilatation and stimulation of LDL oxidation, smooth muscle cell proliferation and monocyte adhesion.

CRP also affects vascular smooth muscle cells (VSMC)\(^ {26}\) by upregulating the angiotensin type 1 receptor (AT\(_1\)-R), which mediates the majority of the proinflammatory effects of angiotensin II. CRP also increases the VSMC proliferation and migration\(^ {26}\).

In summary, CRP seems to unmask an arterial proinflammatory and proatherosclerotic phenotype by (1) reducing nitric oxide synthesis, (2) increasing the release of endothelin-1 and (3) upregulating adhesion molecules and chemoattractant chemokines in endothelial cells and VSMC.
**Monocyte recruitment**

CRP also appears to be involved in recruitment of monocytes, infiltration of monocytes into the vessel wall and subsequent development into foam cells. CRP is deposited in the vessel wall at sites of atherogenesis and has been shown to be chemotactic for freshly isolated human blood monocytes. CRP promotes MCP-1 mediated chemotaxis through upregulation of CC chemokine receptor 2 expression in human monocytes.

**Complement activation**

Another mechanism contributing to CVD is complement activation. CRP is able to activate the classical route of complement activation and it co-localizes with the terminal complement complex in the intima of early atherosclerotic lesions. Griselli et al. demonstrated in an animal model that human CRP and complement activation are major mediators of ischemic myocardial injury. In rats that were injected with CRP infarct size was increased by 40%. Increased levels of complement-CRP complexes are reported in plasma from patients with CVD, indicating that CRP induces activation of complement in vivo. Since complement activation leads to the production of a variety of pro-inflammatory molecules, this is a mechanism by which CRP might aggravate the inflammatory status in the entire body as well as in the atherosclerotic plaque.

In addition to mechanisms that indicate a precipitating role for CVD, also protective functions for CRP in atherosclerosis have been reported. Upon incubation with CRP, endothelial cells from human coronary artery or human saphenous vein show increased expression of complement inhibitory factors. These results suggest that CRP-mediated complement activation is a system set to prevent an inflammatory reaction by promoting the removal of debris from tissues, and the deleterious effects of complement activation in patients with CVD may be the result of derailment of this mechanism in patients with CVD.

**Lipids**

The interaction between lipids and CRP is diverse. It has been suggested that CRP could be the factor linking lipoprotein deposition and complement activation in atherosclerotic plaques. Binding of tissue-deposited CRP to enzymatically degraded LDL (e-LDL) enhances complement activation, which may be relevant to the development and progression of the atherosclerotic lesion, particularly at early stages of atherosclerosis when low concentrations of e-LDL are present.

The reports on interaction between CRP and oxLDL are conflicting, but complement activation as a result of this interaction is generally considered unlikely.
The majority of foam cells below the endothelium show positive staining for CRP\textsuperscript{27}. Zwaka et al\textsuperscript{42} demonstrated that native LDL co-incubated with CRP was taken up by macrophages via macropinocytosis. It was concluded that foam cell formation in human atherogenesis might be caused in part by uptake of CRP-opsonized native LDL.

High levels of high-density lipoprotein (HDL) are atheroprotective since HDL is involved in transporting cholesterol from the periphery to the liver. HDL might also protect the endothelium since the CRP-induced upregulation of inflammatory adhesion molecules in HUVECs was completely blocked by HDL. So, HDL neutralizes CRP induced proinflammatory activity\textsuperscript{43}. HDL also inhibits atherosclerosis through prevention of oxidation of LDL. It is not known whether CRP has an effect on the oxidative status of LDL.

**Thrombosis**

Recently, CRP has also been suggested to directly contribute to CVD by inducing a prothrombotic state. It was reported that CRP directly induces tissue factor expression in human monocytes\textsuperscript{44,45}, but this result could not be confirmed\textsuperscript{46} suggesting that other blood cells may be required to mediate its effect.

Danenberg and colleagues studied the prothrombotic effect of CRP in CRP transgenic mice using a model of transluminal wire injury. They observed that in human CRP transgenic mice 28 days after injury 75\% of the femoral arteries was occluded compared with 17\% in wild-type mice.\textsuperscript{47••} However, like in the study of Paul et al\textsuperscript{18}, plasma CRP levels in the mice were high (18 ±6 mg/l at baseline) and the extrapolation of the results of mice studies to humans should be done with great care.

CRP increases the expression and activity of the main inhibitor of fibrinolysis, plasminogen activator inhibitor-1 (PAI-1) in HAEC\textsuperscript{48}. Since PAI-1 promotes atherothrombosis and progression of acute coronary syndromes, this effect of CRP may also affect CVD\textsuperscript{49}. Indeed, in mice transgenic for human PAI-1 it was recently shown that chronically elevated levels of PAI-1 are associated with age-dependent coronary arterial thrombosis\textsuperscript{50}.

**Angiogenesis/Apoptosis**

CRP might affect CVD progression by inhibiting angiogenesis after myocardial ischemia. Endothelial progenitor cell (EPC) mobilization and differentiation play an important role in angiogenesis and CRP directly inhibits EPC differentiation, survival, and function, partly via reduction of eNOS expression in EPC\textsuperscript{51}. By inhibiting NO production, CRP facilitates endothelial cell apoptosis and blocks angiogenesis\textsuperscript{52}.
Relevance of CRP characteristics

Upon dissociation of its pentameric structure, CRP subunits undergo a spontaneous and irreversible conformational change. Khreiss and colleagues showed that monomeric CRP (mCRP), resulting from the loss of the pentameric symmetry in CRP, is less soluble than CRP, tends to aggregate, and promotes a proinflammatory phenotype of human endothelial cells.\textsuperscript{53} mCRP is a naturally occurring form of CRP and it is a tissue-based rather than a serum-based molecule\textsuperscript{54}. This observation may explain part of the discrepancy in the reported effects of CRP.

Another issue is the purity of the CRP preparations used. Nagoshi et al.\textsuperscript{23} stress the fact that purity of the CRP preparations used to study the protein’s effects on vascular biology is extremely important since removal of endotoxin from commercial rCRP preparations blocked its ability to induce the secretion of IL-6 and MCP-1 by human endothelial cells.

Genetics

It might be considered as the ultimate proof of causality when subjects who are exposed to high CRP their whole life due to genetic predisposition have increased risk of CVD. No environmental factors that determine risk are expected to contribute in this analysis. Very recently, it has become firmly established that a genetic component exists for basal levels of CRP. Baseline levels of CRP show a clear heritability of 40\%\textsuperscript{55} and 39\%\textsuperscript{56} in family studies. In twin studies, MacGregor and colleagues\textsuperscript{57} observed 26\% heritability in middle-aged twins; de Maat et al (unpublished data, 2004) observed heritability of 20\% in elderly twins.

Brull et al\textsuperscript{58} reported the involvement of genetics to CRP, not only with respect to baseline CRP levels, but in particular to the response to stimuli.

Genetic research on CRP can add to knowledge about the mechanisms of involvement of CRP in disease processes that may affect the use of CRP as a marker. Important lessons can be learned from the genetic approach and the consequences of the results.

Szalai et al\textsuperscript{59} reported on a GT repeat polymorphism in intron 1 of the CRP gene. Alleles that are associated with low CRP levels differ in length by exactly 10 bp, which is sufficient for one complete turn of helical double-stranded DNA. This polymorphism disrupts a consensus sequence for the hormone response element HRE-3, suggesting that this polymorphism directly affects the regulation of CRP expression.

Zee and colleagues reported no association between CRP haplotype, consisting of the exonic 1059G/C (dbSNP rs1800947) and the intronic T/A (dbSNP rs1417938) polymorphisms, and venous thromboembolism in a nested case-control sample of the Physicians Health Study\textsuperscript{60}. They had reported previously that the CRP concentrations were significantly reduced among carriers of the 1059C-allele, but the polymorphism was not
associated with risk of arterial thrombosis. A more extensive haplotype analysis in 586 UK simplex systemic lupus erythematosus (SLE) families, including −286C/T/A (dbSNP rs 3091244), 188L/L (1059G/C, dbSNP rs1800947), 988C/T (dbSNP rs1130864), 1846G/A (dbSNP rs1205) and CRP(GT)n, showed that there was a strong linkage disequilibrium within the CRP gene. The rare allele of the 1846G/A polymorphism was associated with the development of SLE. The 188L/L and the 1846G/A polymorphisms made independent contributions to the basal CRP level in these subjects. For the 3′ polymorphism (dbSNP rs1205) this association may be explained by an effect of the variants on mRNA stability. This study could not confirm the previously reported association between the intronic GT dinucleotide repeat and CRP levels.

Another possibility for genetic regulation may be the regulation of the magnitude of response to an inflammatory trigger. Risk factors that directly contribute to CVD will more often be in the dangerous range in hyper-responders than in hypo-responders. It has already been reported by Liuzzo and colleagues that individuals who have a strong response of CRP levels to coronary angioplasty have increased risk of cardiovascular events.

We recently observed that there is a large inter-individual variation in healthy subjects with regard to the response to a mild, standardized inflammatory trigger and that polymorphisms in the promoter region of inflammatory factors predict the increase in plasma levels of the acute phase proteins CRP and fibrinogen. Recently Brull and colleagues reported that the 1444C/T polymorphism in the 3′ region of the CRP gene (988C/T in the paper by Russell et al.) is associated with the increase in CRP levels after coronary artery bypass surgery or after strenuous exercise (Please, note that the nucleotide numbering in the papers varies, and the dbSNP numbers are the unique identifiers of the polymorphisms). These observations suggest that some individuals are genetically predisposed to having a higher response to inflammatory triggers and therefore higher levels of CRP during their life. If CRP directly contributes to CVD, these individuals are expected to be at a higher risk. However, it is expected that for a complex, multifactorial disease one SNP will not show a major contribution to disease risk, and studies taking into consideration environmental factors and variations in other genes in the inflammatory pathway are needed.

**General conclusion**

The causal relationship of CRP with atherosclerosis development and the exact role of each presently reported biological effect in various clinical conditions remains to be established. It is possible as well that CRP influences even more processes before and after ischemia resulting in worsening prognosis of various cardiovascular phenotypes.
The fact that elevated CRP levels are associated with a bad prognosis for patients with CVD, in particular unstable angina or myocardial infarction, is established, but the distinction between a marking role and a biological effect of CRP is important to make, so we can decide about the significance of interventions that reduce circulating levels of inflammatory factors, especially CRP.

*In vitro* studies have shown numerous effects of CRP on endothelial cells, smooth muscle cells, and monocytes; the majority of those effects contribute to proinflammatory and proatherosclerotic effects. CRP affects many cell types involved in atherosclerosis, but the exact mechanism by which CRP contributes to atherosclerosis is still unclear.

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References


   An updated meta-analysis involving a total of 7068 patients with coronary heart disease shows an Odds Ratio of 1.58 (95% confidence interval, 1.48 to 1.68).


- In patients with unstable angina and elevated C-reactive protein in the carotid artery, plaques are less stable which may result in rapid plaque growth and atherosclerotic plaque instability.


- The prothrombotic effect of CRP was shown in CRP transgenic mice using a model of transluminal wire injury, where in the CRP transgenic mice the femoral arteries were occluded more than in wild-type mice 28 days after injury.


