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Review

A role for monomeric C-reactive protein in regulation of angiogenesis, endothelial cell inflammation and thrombus formation in cardiovascular/cerebrovascular disease?

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Summary. Native CRP (nCRP) is a pentameric oligoprotein composed of identical 23 KDa subunits which can be irreversibly dissociated to form free subunits or monomeric CRP (mCRP). mCRP has a reduced aqueous solubility and a tendency to aggregate into matrix-like lattices in various tissues, in particular, blood vessel walls. A dramatic increase in expression of mCRP occurs in angiogenic blood vessels derived from stroked brain regions, atherosclerotic arteries and active vessels from other angiogenic diseases such as Alzheimer's. Furthermore, mCRP unlike the native molecule is highly angiogenic to vascular endothelial cells *in vitro* and therefore might impact on the processes of vascularization and re-modelling thus affecting tissue survival and development. In this mini-review, we will discuss the differences in the biological properties between nCRP and mCRP. We will provide a brief historical background to the importance of nCRP as a biomarker for cardiovascular disease. We will explain the mechanisms of conversion of nCRP to its monomeric form and describe evidence for the role of mCRP in modulation of endothelial cell activation, promotion of inflammatory status and thrombus formation in cardio/cerebrovascular disease. Finally, we will provide evidence for the accumulation of mCRP in angiogenic microvessels from diseased tissue, and demonstrate its highly pro-angiogenic capabilities. The discovery of the existence of this tissue-associated, highly angiogenic monomeric form of CRP capable of cellular binding and intra-cellular signal transduction activation may help in our understanding of the processes responsible for modulation of angiogenesis and inflammation in disease.

Key words: C-reactive protein, Angiogenesis, Monomeric, Signal transduction

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Native CRP (nCRP) expression in vascular disease

Much of the original published data on CRP has established its increased expression as a general marker of inflammatory disease. High concentrations of nCRP are related to increased risk of vascular episodes, and correlated with brain infarct area, with the severity of ischemic episodes, with greater neuronal damage and with a higher risk of future vascular episodes (Smith et al., 2004; Krupinski et al., 2007). Increased nCRP levels in the plasma arise due to enhanced synthesis by the liver, as a result of interleukin-6 (IL-6) induction. It forms part of a non-specific acute response of the organism to inflammation, infection and tissue damage; for this reason, it was long considered that measurement of systemic levels could not add useful clinical information (Sepulveda and Mehta, 2005; Pepys et al., 2006). However, recent studies have demonstrated an important role for nCRP in atherosclerosis and ischemic vascular disease. Although there has been some controversy about the predictive value of nCRP, for example, in the Reykjavik study of 18,569 patients which included 2459 with nonfatal myocardial infarction, and in the NPHS-II and EAS studies the data suggested that nCRP may be only a moderate predictor of coronary heart disease, not adding to the Framingham risk score (Danesh et al., 2004; Shah et al., 2008), many other studies have demonstrated that analysis of high

sensitivity (HS) nCRP plasma levels can predict cardiovascular events better than LDL cholesterol levels, and systemic plasmatic activation associated with inflammation is associated with coronary atherosclerosis and impaired circulation, thus adding a prognostic value to the Framingham's risk punctuation (Ridker et al., 2005; de Ferranti and Rifai 2007; Brunetti et al., 2008). Recent evidence is therefore emerging that nCRP may in fact be an important biomarker, able to predict the pathogenesis of atherosclerosis.

Native CRP (nCRP) versus modified CRP (mCRP) in diseased tissue

CRP is a pentameric oligoprotein composed of 5 identical 23 KDa subunits which can be irreversibly dissociated to form free subunits or mCRP (Fig. 1). mCRP has a reduced aqueous solubility and a tendency to aggregate into matrix-like lattices in various tissues, in particular, blood vessel walls (Diehl et al., 2000). Cell membranes and liposomes dissociate nCRP to form this more highly biologically active derivative (Ji et al., 2007). A distinct difference in the biological activity of these two isoforms has been shown. mCRP is the primary isoform which binds native and modified low/density lipoproteins, it is the most effective activator of the complement cascade when bound to LDL or oxidised LDL, however, fluid phase mCRP can bind to and prevent C1q from subsequent down-stream activation of the complement cascade. mCRP can therefore both inhibit and activate the classical complement pathway by binding C1q, depending on whether it is in fluid phase or surface-bound state.

mCRP promotes activation and inflammatory cytokine production in vascular endothelial cells and increases platelet adhesion and thrombus growth directly

Previously published studies have also shown that mCRP can induce EC activation at concentrations significantly below the CVD risk cut off point of 3.7 (i.e. approximately 1 µg per ml) (Ji et al., 2006). Using carefully purified and synthesised mCRP and nCRP, Bogulawski et al. (2007), showed that only FITC-labelled mCRP was able to bind to IgG molecules including pentraxins and vitronectin, again, suggesting alternative and increased activity of the modified form. Khreiss et al. (2004), demonstrated that only mCRP could significantly increase gene expression of IL-8 and MCP-1 within 4h through a p38 MAP kinase dependent pathway in HCAEC at physiological concentrations (1 µg/ml). Vascular EC can directly express and secrete CRP (Jabs et al., 2003). *In vitro* studies using EC and VSMC cultures, also demonstrated increased de novo expression of CRP in response to various inflammatory stimuli (Calabro et al., 2003; Venugopal et al., 2005) and mCRP following hypoxia (Slevin et al., 2009). In the first studies (response to inflammatory stimuli) commercial antibodies used recognised both the native form of and mCRP and so the relative expression of each was not determinable. However, they did demonstrate a

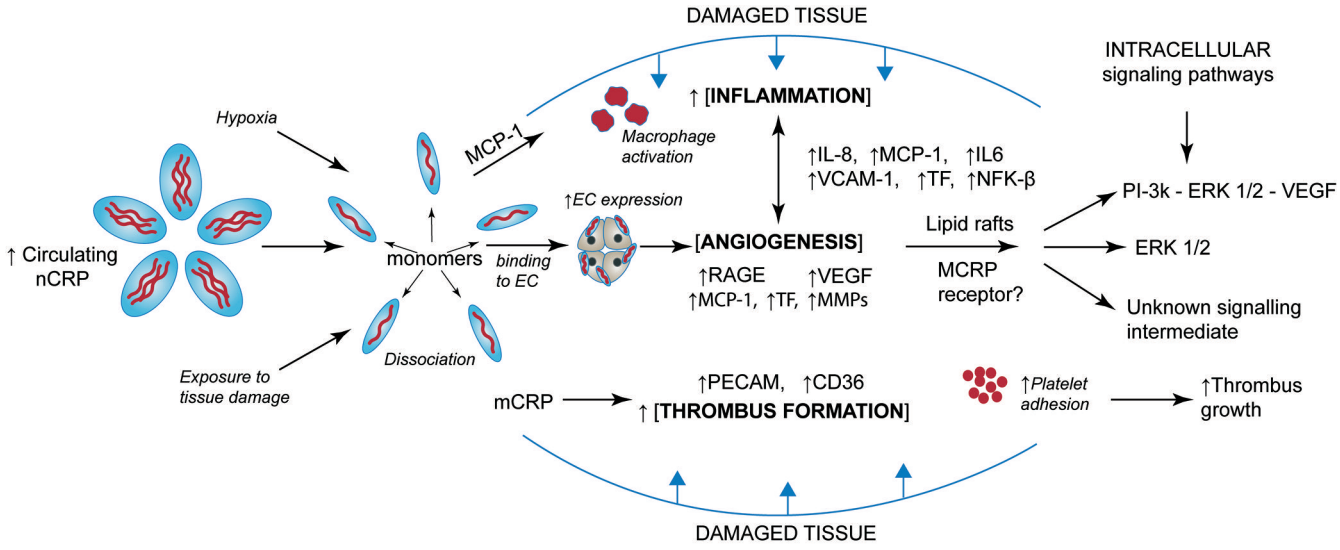

Fig. 1. The pentameric pentraxin nCRP can dissociate rapidly when in contact with tissues and cells, particularly in the presence of hypoxia and necrosis forming individual monomers which can bind to vascular and inflammatory cells and perpetuate inflammation and angiogenesis through stimulation of cytokine production and initiation of intra-cellular signal transduction pathways through as yet, undetermined receptor-mediated mechanisms.

concomitant rise in IL-6 (main inducer of CRP expression) and of the chemotactic protein MCP-1 (a protein induced by CRP), which might reflect a mechanism through which CRP of vascular origin contributes to maintaining and promoting the inflammatory process in diseased tissue. Most recently, Ji et al. (2009) demonstrated that purified mCRP was able to insert into the membrane rafts of vascular EC where it activated cytokine release (including interleukin-8), reactive oxygen species generation and adhesion molecule expression. Intra-cellular signalling and cytokine production was dependent on maintenance of normal raft conformation since disruption with methyl-beta cyclodextrin inhibited mCRP-induced IL-8 release suggesting this as a mechanism for intracellular signal transduction associated with perpetuation of the inflammatory response.

In relation to other possible effects of mCRP associated with plaque instability and thrombosis, it is worthy of mention that mCRP (5 µg/ml) but not nCRP was able to significantly increased platelet adhesion and thrombus growth when directly incubated with blood and when immobilized on a collagen surface. Confocal immunohistochemistry revealed the presence of mCRP on the surface of adhered platelets and within the thrombus and showed an up regulation of P-selectin and CD36 in effluent platelets pre-incubated with mCRP. Flow cytometric analysis of collagen-induced platelet activation demonstrated that only mCRP, significantly increased platelet surface P-selectin expression without modifying CD63 and PAC-1. Our data indicate that whereas serum nCRP may not affect thrombus growth, mCRP displays a pro-thrombotic phenotype enhancing not only platelet deposition, but also thrombus growth under arterial flow conditions (Mollins et al., 2008).

Defining the role of nCRP/mCRP in modulating angiogenesis has been hampered by contaminants found in commercial preparations

Some earlier studies attributed functions to CRP which may have been due to contamination of the original commercial preparations by endotoxins and/or azide. For example, sodium azide in commercial CRP preparations was found to be responsible for a reduction in proliferation, migration and tube formation in matrigel as well as to induce pro-apoptotic effects in human umbilical vein EC (HUVEC) (Liu et al., 2005; Taylor et al., 2005). In more recent studies the presence of lipopolysaccharides (LPS) and azide was strictly controlled, and in most cases not present. In experimental in vitro studies, extreme care has to be taken to confirm their validity by conducting analysis using only purified endotoxin-free, azide-free human recombinant CRP preparation, thus ensuring that the effects are not due to either lipopolysaccharide/bacterial

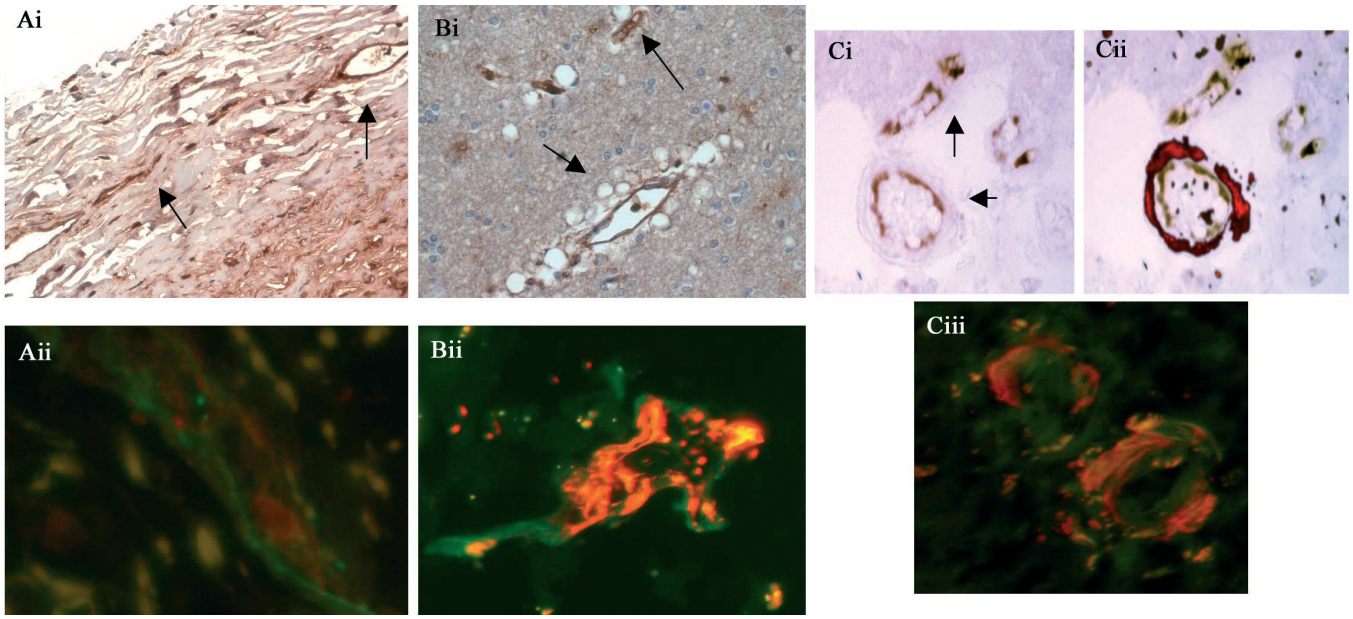

Fig. 2. Ai and **Bi** and **Ci** respectively show low power views of mCRP stained adventitial vasa vasorum from a grade V1 carotid lesion, peri-infarcted stroke tissue and vessels in the vicinity of amyloid plaques from a patient with Alzheimer's disease (DAB brown; x 40; arrows). **Aii** and **Bii** show co-immunoflourescent staining in the same samples with mCRP (red; TRITC) and CD105 (green; FITC; x 200) **Cii** shows Alzheimer´s brain microvessels stained with anti-ß-amyloid antibodies (red; TRITC) and anti mCRP antibodies (DAB brown; x 100) and **Ciii** shows the same vessels co-immunoflourescently stained with antibodies directed against ß-amyloid (stained green; FITC) and mCRP (stained red; TRITC; x 100).

product contamination. SDS-PAGE electrophoresis can be used to confirm purity. Endotoxins should be continuously monitored using the limulus assay (Chromogenix, Molndal, Sweden), and if identified (>10pg/ml), fresh preparations allocated.

Previous studies have shown that purified commercially obtained CRP, free of azide and LPS were able to up-regulate potentially angiogencic proteins including the receptor for advanced glycation end products in human saphenous vein EC (Zhong et al., 2006), nuclear factor-κB, and vascular cell adhesion molecule-1 in HUVEC and human aortic EC (HAEC) (Liang et al., 2006) and tissue factor expression together with EC proliferation and activation of ERK1/2 in thoracic and coronary derived rabbit EC (Cirillo et al., 2005). Similarly, CRP also induced matrix metalloproteinase-1/10 gene and protein expression through p38 and MEK and p38 and c-jun N-terminal kinase signalling pathways respectively (Montero et al., 2006). Montero et al. also showed in the same study that increased CRP and MMP-10 co-localized in EC and macrophage rich areas and was associated with intina-media thickness of carotid arteries suggesting a strong link between their expression, angiogenesis, inflammation and increased plaque vulnerability. Dasu et al. (2007) demonstrated that CRP, obtained from pleural fluid, stimulated interleukin-6/8/1beta, plasminogen activator inhibitor-1 and endothelial nitric oxide synthase in control and toll-like receptor-4 HAEC knockdowns in contrast to LPS. nCRP induced BAEC insulin resistance via CD64-receptor activated stimulation of JNK, RhoA, Syk tyrosine kinase and insulin receptor substrate-1 (Xu et al., 2007). Similarly, Bello et al. (2008) recently showed that azide/LPS purified commercially obtained CRP induced vascular endothelial cell growth factor-A (VEGF-A) expression via phosphatidyl-inositol-3-kinase (PI-3K) and early response kinase 1/2 (ERK1/2) activation. However, in the above studies, the authors did not determine if their samples contained mCRP which could also have been responsible for these effects.

More recent studies have employed purification of commercial sources of nCRP through phenyl sepharose columns (to remove traces of mCRP) before comparing the angiogenic effects with those produced by mCRP.

Our recent published studies showed that commercial samples of nCRP induced a significant increase in bovine aortic EC (BAEC) and human coronary artery EC (HCAEC) migration and tube formation in matrigel; they also induced rat aortic ring vascular sprouting as well as vessel formation in the chick chorioallantoic membrane at concentrations of 1 μg/ml and higher with a potency similar to that of FGF-2 (Turu et al., 2008). We have subsequently shown that removal of traces of mCRP from this CRP nullified its angiogenic effects demonstrating that mCRP but not pure nCRP is angiogenic. mCRP also induced phosphorylation of ERK1/2, a key angiogenic/mitogenic signalling intermediate, and pharmacological inhibition with PD98059 or an antibody fully characterized and specific to mCRP was sufficient to prevent angiogenesis (Slevin et al., 2009). As mentioned above, the ability of mCRP to bind to vascular EC and anchor within lipid rafts suggests a possible mechanism through which mCRP may also promote angiogenesis but this needs to be investigated in more detail (Ji et al., 2009).

Tissue localization of mCRP in angiogenic disease

We have shown that mCRP rather than nCRP is over-expressed in neovessel-rich, CD105-positive areas of peri-infarcted brain tissue (Slevin et al., 2009), associated with peripheral blood mononuclear cells and neovessels in hypoxic areas of cardiac tissue following porcine experimental myocardial infarction (Vilahur et al., 2008) in active, CD105-positive vasa vasorum and intimal neovessels in complicated carotid atherosclerotic lesions (Submitted for publication) and in beta amyloid-expressing vessels from patients with Alzheimer's disease (Fig. 2). These finding are supported by the published work of Ji et al. (2007, 2009) who demonstrated that under pathological conditions, nCRP binds to cell membranes in a calcium-dependent manner and undergoes a partial structural change forming $mCRP_m$ retaining pentameric configuration but with enhanced sub-unit activity, followed by detachment from the membrane and conversion to mCRP which has potent activatory effects on EC.

These studies indicate that mCRP may be an important regulator of signalling pathways associated with both angiogenesis and inflamation. Knowledge of the cellular and molecular mechanisms through which mCRP promotes activation of vascular EC and angiogenesis may contribute to the design of novel therapeutic treatment aimed at reducing inflammation and blocking neovessel formation in atherosclerotic plaques. Characterised proteins and signalling molecules associated with mCRP signalling and induction of inflammation and/or angiogenesis are shown in Figure 1. Alternatively, in vascular angiogenic diseases such as stroke and myocardial infarction, promotion of vascularization and reperfusion might help to optimise the recovery of tissues subjected to ischemia (Slevin et al., 2006, 2009; Doyle and Caplice, 2007).

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