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The connection between C-reactive protein and atherosclerosis

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Abstract

The connection between C-reactive protein (CRP) and atherosclerosis lies on three grounds. First, the concentration of CRP in the serum, which is measured by using highly sensitive (a.k.a. 'hs') techniques, correlates with the occurrence of cardiovascular disease. Second, although CRP binds only to Fc γ receptor-bearing cells and, in general, to apoptotic and damaged cells, almost every type of cultured mammalian cells has been shown to respond to CRP treatment. Many of these responses indicate proatherogenic functions of CRP but are being reinvestigated using CRP preparations that are free of endotoxins, sodium azide, and biologically active peptides derived from the protein itself. Third, CRP binds to modified forms of low-density lipoprotein (LDL), and, when aggregated, CRP can bind to native LDL as well. Accordingly, CRP is seen with LDL and damaged cells at the atherosclerotic lesions and myocardial infarcts. In experimental rats, human CRP was found to increase the infarct size, an effect that could be abrogated by blocking CRP-mediated complement activation. In the *ApoB*^{100/100}*Ldlr*^{-/-} murine model of atherosclerosis, human CRP was shown to be atheroprotective, and the importance of CRP-LDL interactions in this protection was noted. Despite all this, at the end, the question whether CRP can protect humans from developing atherosclerosis remains unanswered.

Keywords

Atherosclerosis; cholesterol; C-reactive protein; foam cell; low-density lipoprotein; phosphoethanolamine; myocardial infarction

Introduction

C-reactive protein (CRP) is a pentagonal doughnut-shaped acute phase protein whose five identical subunits are arranged in cyclic pentameric symmetry (1,2). The abbreviation CRP denotes native pentameric CRP whose concentration in biological fluids is measured by using highly sensitive techniques. Although it has never been felt necessary to associate the name of the technique with the name of the protein, CRP is frequently called high-sensitivity CRP (hsCRP). In this article, we use the abbreviation CRP and not hsCRP. Research in the last many years has contributed greatly to our knowledge regarding the possible involvement of CRP during the development of atherosclerosis. Atherosclerosis is a disease caused by the deposition and modifications of low-density lipoprotein (LDL) in artery walls. Modified LDL is engulfed by macrophages to form foam cells that contribute to the development of atherosclerosis (3,4). CRP was discovered in 1930, and within the next three decades, data on the interaction of CRP with lipids indicating a connection between CRP

and atherosclerosis were published (5,6). In this review, we begin there and end with the latest reports on the targeting of CRP for the treatment of cardiovascular disease and on the atheroprotective role of CRP seen in a mouse model of human atherosclerosis (7,8). Numerous proinflammatory and proatherogenic functions have also been suggested for CRP, but most of them are not universally accepted yet (9–11). We discuss this aspect of CRP too and propose a precaution to take for generating reproducible results in experiments using pure preparations of CRP.

Biosynthesis and clinical value of CRP

The life history of CRP begins with its biosynthesis in the liver followed by secretion into the circulation (12). The production of CRP is increased in atherosclerosis and other cardiovascular diseases which involve low-grade systemic inflammation (13–16). Using primary human hepatocytes and hepatoma cells, it has been shown that cytokines interleukin (IL)-6, IL-1 β and IL-17 induce CRP gene expression (12,17–19). The increased production of CRP results in a rise in its serum concentration. The half-life of CRP in human circulation is about 19 h (20). In response to inflammatory mediators, low-level expression of CRP in cells other than hepatocytes has also been observed (21–23). The extrahepatic CRP-expressing cells include atherosclerotic plaque tissue, monocytes, aortic endothelial cells, and vascular smooth muscle cells (21–28).

The median concentration of CRP in the general population of normal healthy individuals is 0.8 mg/L. A concentration of CRP ranging from 3 mg/L to 10 mg/L is considered slightly elevated (20,29). Epidemiological studies have shown that the individuals who developed cardiovascular disease had slightly elevated serum CRP levels (30,31). Although it is not known whether CRP can afford these patients a longer life, but because of the correlation between serum levels of CRP and occurrence of cardiovascular disease, the American Heart Association and the Center for Disease Control recommended physicians to routinely measure CRP along with cholesterol levels to predict the risk of future atherosclerotic events (32–34).

Statins, the inhibitors of a key enzyme in the cholesterol biosynthesis pathway, are used in humans as cholesterol-lowering drugs (35). However, statins also lower CRP levels in humans and in IL-1 β -treated human CRP-transgenic mice (30,31,36,37). It has been shown that statins act directly on hepatocytes or hepatoma cells and prevent cytokine-mediated induction of CRP expression (37–39). Similarly, fibrates and the nitric oxide donor, sodium nitroprusside, also prevent induction of cytokine-induced CRP expression in hepatic cells (37,38,40). The clinical value of CRP therefore may be diminished in patients taking these agents which inhibit biosynthesis of CRP even if proinflammatory cytokines are present (38).

Dissociation of pentameric CRP into monomeric CRP

Although CRP is secreted by hepatocytes in the form of pentameric molecules, it is susceptible to modifications both *in vitro* and *in vivo*. *In vitro*, while stored under nonphysiological conditions such as in the absence of calcium, the monomers in CRP are slowly dissociated by a nonproteolytic and irreversible process and release the monomeric form of CRP (mCRP) (41,42). In the absence of calcium, both CRP and mCRP are susceptible to proteolytic degradation (41,43,44). Thus, CRP should always be stored in the presence of calcium. However, it should be noted that a concentration of calcium above 2 mM causes aggregation of CRP, and a concentration of calcium above 1 mM causes precipitation of CRP in phosphate buffers (45, and unpublished observations). If it becomes necessary to store CRP without calcium, then CRP should be repurified by a quick high-

performance liquid chromatography gel filtration procedure to recover native pentameric CRP just before use in the experiments.

The mCRP can also be generated by treating pentameric CRP with protein denaturants or by genetic engineering of CRP cDNA (46,47). *In vivo*, the presence of mCRP has been demonstrated in both normal vascular tissue and atherosclerotic lesions (47,48). A mechanism for the processing of CRP into mCRP *in vivo* has been proposed: when CRP is associated with a membrane, it is converted to mCRP (49,50). In mice, the half-life of mCRP in the circulation is less than 5 min compared to approximately 4 h for pentameric CRP; mCRP is rapidly cleared from the circulation and migrates to tissues (51).

There are many differences between mCRP and CRP in their structure, ligand recognition properties, and effector functions (41,42,47,52–57). Many laboratories and most clinics use commercially available CRP measurement kits to determine the concentration of CRP in serum. Some kits use polyclonal anti-CRP antibodies that recognize CRP irrespective of the different structural states of CRP. Thus, the levels of CRP measured by only polyclonal antibody-based methods reflect not only pentameric CRP but also mCRP. Because there is no evidence from clinical trials that it is necessary to make a distinction between different forms of CRP *in vivo*, and because the concentration of mCRP in serum could be negligible, it is not necessary, at present, for the CRP measurement kits to include monoclonal antibodies specific for different forms of CRP to determine the concentration of CRP in serum.

Binding of CRP to native and modified LDL

Preliminary evidence for the interaction of CRP with lipids indicating a possible relationship between CRP and atherosclerosis came from the finding that CRP had a lipid-flocculating property (6,58). The lipid-flocculating property of CRP was due to the interaction between CRP and cholesterol (59). The interaction between CRP and cholesterol was confirmed subsequently (60,61). *In vitro*, native pentameric CRP does not bind to native LDL; however, it does bind to modified forms of LDL such as oxidized LDL (ox-LDL) and enzymatically modified LDL (E-LDL) in a Ca^{2+} -dependent and reversible manner (60–67). Aggregated forms of native pentameric CRP have been shown to bind to native LDL as well in whole serum (62). *In vitro*, native CRP and native LDL interact only when either one is immobilized or modified (45,63,65,66). The binding of CRP to ox-LDL has also been demonstrated *in vivo* in diabetes mellitus patients with atherosclerosis (68). The binding of CRP to traces of very low-density lipoprotein (VLDL) from whole serum has also been shown (62,69,70).

The binding site on CRP for LDL has been explored. One of several thoroughly investigated ligand-binding sites on CRP is the phosphocholine (PCh)-binding site for its oldest known ligand PCh (71–76). There are five PCh-binding sites in CRP, one on each monomer (1). Besides LDL cholesterol, CRP binds to many PCh-containing substances such as pneumococcal C-polysaccharide and damaged or altered cell membranes of apoptotic and necrotic cells (77–81) and also to many non-PCh ligands such as phosphoethanolamine (PEt), galactose-containing substances, extracellular matrix protein fibronectin, and complement factor H (44,74,82–90). Some of these reactions of CRP require prior binding of calcium ions to CRP and occur through the PCh-binding site of CRP. The participation of the PCh-binding site of CRP in binding to modified LDL has been demonstrated by the inhibition of binding of CRP to modified LDL by PCh (60,62–65). The moieties on the LDL molecule that interact with CRP include ApoB, cholesterol and PCh (60,61,63,65,91).

Although the binding of CRP to E-LDL is mediated by the PCh-binding site in CRP, our mutagenesis studies have revealed that the amino acids in CRP that contact PCh are not

critical for binding to E-LDL, indicating that the PCh groups in E-LDL are not necessary for CRP–E-LDL interaction. We also found that the blocking of the PCh-binding site of CRP with PEt, but not with PCh, changed the LDL-binding property of CRP. The binding of PEt-complexed CRP to E-LDL was dramatically enhanced over that of uncomplexed CRP. PEt-complexed CRP also acquired a new function: it bound selectively to native LDL in whole serum. The mechanism of action of PEt is not known, but considering the fact that aggregated CRP binds to native LDL it can be assumed that PEt might cause aggregation of CRP. As expected, PEt inhibited the binding of CRP to other PCh-containing noncholesterol substances (unpublished observations). These findings raise the possibility that the administration of PEt-based compounds to target endogenous CRP to form PEt-CRP complexes or the administration of exogenously prepared PEt-CRP complexes may be useful to capture native and modified LDL *in vivo* and, at the same time, to prevent binding of CRP to other PCh-containing substances such as damaged cells at the myocardial infarcts.

The mCRP binds to both native and modified LDL and thus low levels of mCRP contaminant could confer native CRP obvious native LDL-binding capacity. It has been proposed that mCRP may exert a protective role by facilitating the clearance of retained native LDL from extracellular space, and thus lower the risk of atherogenic LDL derivative formation, and that the interaction of mCRP with LDL may contribute to the regulation of LDL metabolism (92).

CRP and the formation of LDL-loaded macrophage foam cells

Because CRP binds modified forms of LDL, the possibility that CRP might play a role in the uptake of modified LDL by macrophages has been investigated from time to time. In initial studies, the effect of CRP on the uptake of LDL by macrophages was examined using mixtures of CRP and LDL for the treatment of macrophages. These studies, employing antibodies to CRP, antibodies to ApoB, or labeled LDL, revealed that CRP did not prevent uptake of modified LDL by macrophages (45,93,94). When measured indirectly by Fc γ receptor IIa (Fc γ RIIa, CD32) internalization, CRP was found to increase LDL uptake; however, the involvement of Fc γ RIIa was questioned (45,95). Whether CRP is present inside the cytoplasm of macrophage foam cells located in the atherosclerotic lesions is not known (96–98).

We recently investigated the effect of CRP on the accumulation of lipid droplets made up of cholesteryl esters in E-LDL-treated macrophages, which is a hallmark of foam cell formation. We found that, in contrast to E-LDL alone, the CRP-bound E-LDL was inactive in the formation of foam cells. This function of CRP required that E-LDL must be bound to CRP. The mere presence of CRP with E-LDL was not sufficient to prevent foam cell formation. Thus, an inherent function of CRP is to prevent E-LDL-induced formation of macrophage foam cells. Because PEt-CRP complexes bind to E-LDL more efficiently than uncomplexed CRP, it may be possible to enhance the function of CRP to prevent foam cell formation, by administering PEt-based compounds to target endogenous CRP to form PEt-CRP complexes or by administering exogenously prepared PEt-CRP complexes (99, and unpublished observations).

The mCRP has also been shown to decrease uptake of ox-LDL by macrophages, and it has been proposed that the interaction of mCRP with ox-LDL may contribute to retardation of the foam cell formation by reducing the aggressive macrophage response to ox-LDL (92). A preventive role in foam cell formation has also been reported for serum amyloid P (SAP), a protein which in many ways is similar to CRP. SAP binds to ox-LDL because ox-LDL contains amyloid structures. The SAP-bound ox-LDL was not taken up by macrophages (100).

Effects of CRP on cultured mammalian cells

Almost every type of cultured mammalian cells, including vascular cells, has been shown to respond to CRP treatment. Many of the responses of vascular cells to CRP indicate proinflammatory and proatherogenic functions of CRP (9–11,101). In addition to modulating the physiology of cultured cells, CRP also affected the growth of cells. For example, in vascular smooth muscle cells, CRP induced apoptosis (102) in one study and increased cellular proliferation in another (103). In endothelial cells, CRP triggered intracellular signaling to activate transcription factors such as NF- κ B (nuclear factor-kappa B) and GADD153 (growth arrest and DNA damage-inducible protein 153) and the p38 MAPK (mitogen-activated protein kinase) pathway, and increased the expression of several genes (102–110). The antiatherosclerosis drugs statins and fibrates reduce proinflammatory functions of CRP in endothelial cells (109–112).

The results obtained from cell culture experiments using purified CRP remain a subject of concern because of the presence of sodium azide and endotoxins in CRP preparations. The main reason for the confusion about the role of CRP in atherosclerosis is that in many studies recombinant *Escherichia coli*-expressed, azide-containing CRP was used and that the results observed could be ascribed to the contamination with bacterial products and azide rather than to CRP itself (113–117). Thus, it is not established that CRP is a proatherogenic molecule (118). Indeed, it has been confirmed that some, if not all, of these *in vitro* functions of CRP were not due to azide and endotoxins (119–121). But because of the controversies, it is best to use CRP or mCRP free of azide and endotoxins for both *in vitro* and *in vivo* experiments.

Most preparations of CRP are labeled as pure because they do not contain a protein contaminant. It is important to note that, in addition to nonprotein contaminants, another major contaminant in CRP preparations is derived from CRP itself. Almost all CRP preparations contain degraded CRP and aggregated CRP generated due to storage conditions (41,92). CRP dissociates into monomers and degrades into peptides which may form aggregates also. More recent reports have indicated that mCRP was a prerequisite for CRP to exert stimulatory effects on endothelial cells (55). Because the peptides and aggregates of CRP also modulate cellular activities and may have profound effects in cell culture (56,122–130), it is critical that purification of CRP using a quick gel filtration method be repeated immediately before using CRP for the treatment of cells. The differences in the contents of these contaminants in various preparations of CRP are likely to generate irreproducible results.

Cells respond to CRP-treatments, but there is lack of data on the binding of CRP to all kinds of responsive cells. The binding of CRP has been described only for the endothelial cells and, in general, for apoptotic cells and Fc γ R-bearing cells (78–81,131–134). The binding of CRP to Fc γ R had been debated some years ago (135–137), but in the last 5 years or so, several laboratories have reported Fc γ R-mediated functions of CRP (56,138–147). Apparently, an experimental condition can be set where CRP will exhibit its Fc γ R-binding capability. The binding of CRP to endothelial cells is mediated by Fc γ R present on these cells, and the binding of CRP to apoptotic cells is mediated by PCh groups present on these cells (65,106,142,143). It is not known whether the binding of CRP to a very small percentage of apoptotic cells in culture would change the physiology of nonapoptotic cells. So far, comparative studies involving recombinant mutants of CRP incapable of binding to cells through Fc γ R or through PCh have not been conducted to confirm cellular responses to CRP.

Functions of CRP in atherosclerosis in mice

CRP has been found deposited and, importantly, colocalized with LDL and macrophages in atherosclerotic lesions in humans and experimental animal models (64,98,148,149). The presence of CRP at the lesions was not unexpected because of the known interaction between CRP and LDL *in vitro*. Although local synthesis of CRP from arterial cells and macrophages has been shown, a recent report indicated that CRP present at the atherosclerotic lesions was transported from the circulation (98). To determine the role of CRP in the development of atherosclerosis, human CRP has been used in various mouse models of atherosclerosis (8,150–158).

Initially, CRP was found to be proatherogenic. There was modest acceleration in aortic atherosclerosis in male *ApoE*^{-/-} mice expressing high levels of CRP (150). That CRP increased atherosclerosis in mice was also shown in another independent study (151). CRP was also shown to be proatherogenic in hypercholesterolemic humans (152,153).

Subsequently, CRP was found to be neither proatherogenic nor atheroprotective in *ApoE*^{-/-} mice (154–156). A limited amount of passively administered human CRP was not atheroprotective in *ApoE*^{-/-} mice (151). Similarly, transgenic human CRP was also not atheroprotective in *ApoE*^{-/-} mice (154–156). Thus, the cell culture-based results implicating CRP as a pathogenetic factor in atherosclerosis could not be extended to *in vivo* situations. Additionally, it was shown that the lowering of CRP was not required to reduce atherosclerosis in mice transgenic for human CRP; just the lowering of cholesterol was sufficient (157,158). The presence of CRP did not affect the efficacy of statins in reducing atherosclerosis indicating that CRP was not proatherogenic (157,158). Interestingly, mCRP has been shown to be atheroprotective in mice (151).

Recently, CRP was found to be atheroprotective in atherosclerosis-prone *Apob*^{100/100}*Ldlr*^{-/-} mice. These mice are rich in LDL and develop hypercholesterolemia in a more human-like manner (8). These findings indicated that the binding property of CRP to LDL may play a role in slowing the development of atherosclerosis in these mice. It was suggested that the *ApoE*^{-/-} mouse model of atherosclerosis was not the most appropriate model for investigating the functions of CRP because *ApoE*^{-/-} hypercholesterolemic mice were rich in VLDL and not in LDL (8). The use of *ApoE*^{-/-} mice in determining the role of human CRP in the development of atherosclerosis is not appropriate for one more reason: the differences in the ability of human CRP to activate the complement system in human and mouse serum. In human serum, after complexing with an appropriate ligand, human CRP activates the classical pathway of complement (76). However, human CRP does not activate complement in serum from *ApoE*^{-/-} mice (155,159). Indeed, the deposition of complement C3 in the atherosclerotic lesions in these mice, and also in *Apob*^{100/100}*Ldlr*^{-/-} mice, was not different in the presence and absence of human CRP (8,154).

Since CRP is only a trace serum protein in mice, they are commonly used to investigate the functions of human CRP. But, recent data have shown that CRP exhibits species specificity. In humans, human CRP binds complement C1q to activate the classical complement pathway and binds lectins to activate the lectin complement pathway (76,160,161). In mice, although human CRP activates the lectin pathway, it does not bind mouse C1q and therefore cannot activate the classical complement cascade (160). Thus, mouse is not suitable for exploring any function of CRP that may involve CRP-mediated activation of the classical complement pathway. It is important to establish the *in vitro* properties of CRP using mouse materials so that experiments to determine *in vivo* functions of human CRP can be interpreted in mice.

CRP and myocardial infarction

Atherosclerosis leads to myocardial infarction. Since CRP binds to injured cells, CRP gets deposited at myocardial infarcts (16,162–164). To define the functions of CRP in myocardial infarction, rats have been employed. Rat CRP does not activate its own complement, but human CRP does activate rat complement. Injection of human CRP into rats undergoing experimental myocardial infarction increased the infarct size, and this was due to the complement-activating property of CRP-complexes (165,166). Although rats have their own CRP and capable of binding to damaged cells, rat CRP did not enhance the infarct size because rat CRP did not activate its own complement (166). Possible interaction between human CRP and rat CRP and possible competition between the two proteins for binding to damaged cells in the rats are not known. CRP also increased thrombosis and arterial occlusion in a mouse model of vascular injury (167). Although the activation of complement by CRP deposited at the infarcts is not beneficial, the CRP-mediated modulation of complement activation by E-LDL could be protective (159).

CRP can have both proinflammatory and anti-inflammatory activities (168,169). In myocardial infarction in rats, CRP has proinflammatory activity which is dependent on complement activation and is detrimental. Under conditions of atherosclerotic plaque formation, when low levels of CRP and low levels of complement are present, the activity of CRP will most likely be anti-inflammatory and is beneficial (8,99,159). A PCh-based compound was recently reported to inhibit the deposition of human CRP at the rat myocardial infarcts and inhibit the subsequent activation of the rat complement (7). However, such a compound should only be used in the acute events because these compounds might lessen the beneficial anti-inflammatory effects of CRP on atherosclerosis. Therefore, we suggest the use of PEt-based compounds because these compounds will block the binding of CRP to myocardial infarcts and therefore block the proinflammatory activity of CRP. Simultaneously, PEt-based compounds will enhance the anti-inflammatory activity of CRP in atherosclerosis by enhancing the binding of CRP to LDL.

Future directions: pharmacologic intervention of CRP to prevent atherosclerosis

Because human CRP has been shown to be atheroprotective in the mouse model rich in LDL but not in the mouse model rich in VLDL, the capability of CRP to bind to LDL could be further exploited therapeutically to enhance the atheroprotective effects of CRP. The capacity of aggregated CRP to bind to native LDL may be exploited to capture serum LDL cholesterol. The capacity of native CRP to bind to modified LDL may be exploited to prevent the formation of LDL-loaded macrophage foam cells. Pharmacologic intervention of CRP with small molecules such as PEt to partially block the PCh-binding site of CRP may cause aggregation of CRP and enhance binding of CRP to both native and modified LDL. Although the fate of the CRP-bound LDL needs to be determined, possibly CRP-bound LDL would be catabolized or prevented from further modifications (170,171). In conclusion, experiments using *Apob*^{100/100} *Ldlr*^{-/-} mice should be pursued to investigate whether CRP can protect these mice from developing atherosclerosis under the conditions that enhance interaction of CRP with LDL molecules.

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Abbreviations

CRP	C-reactive protein
mCRP	monomeric CRP
PCh	phosphocholine
PEt	phosphoethanolamine
Apo	apolipoprotein
LDL	low-density lipoprotein
E-LDL	enzymatically modified LDL
ox-LDL	oxidized LDL

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Key messages

- Human C-reactive protein (CRP) has been shown to be atheroprotective in the *ApoB^{100/100}Ldlr^{-/-}* mouse model of human-like hypercholesterolemia.
- The capacity of aggregated CRP to bind to native low-density lipoprotein (LDL) may be exploited to capture serum LDL cholesterol.
- The capacity of native CRP to bind to modified LDL may be exploited to prevent the formation of LDL-loaded macrophage foam cells.
- To obtain reproducible data, CRP should be free of endotoxins and sodium azide and should be freshly purified to avoid contamination with the degradation products derived from the protein itself.