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Atherosclerosis-related functions of C-reactive protein

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Abstract

C-reactive protein (CRP) is secreted by hepatocytes as a pentameric molecule made up of identical monomers, circulates in the plasma as pentamers, and localizes in atherosclerotic lesions. In some cases, localized CRP was detected by using monoclonal antibodies that did not react with native pentameric CRP but were specific for isolated monomeric CRP. It has been reported that, once CRP is bound to certain ligands, the pentameric structure of CRP is altered so that it can dissociate into monomers. Accordingly, the monomeric CRP found in atherosclerotic lesions may be a stationary, ligand-bound, by-product of a ligand-binding function of CRP. CRP binds to modified forms of low-density lipoprotein (LDL). The binding of CRP to oxidized LDL requires acidic pH conditions; the binding at physiological pH is controversial. The binding of CRP to enzymatically-modified LDL occurs at physiological pH; however, the binding is enhanced at acidic pH. Using enzymatically-modified LDL, CRP has been shown to prevent the formation of enzymatically-modified LDL-loaded macrophage foam cells. CRP is neither pro-atherogenic nor atheroprotective in *ApoE*^{−/−} and *ApoB*^{100/100}*Ldlr*^{−/−} murine models of atherosclerosis, except in one study where CRP was found to be slightly atheroprotective in *ApoB*^{100/100}*Ldlr*^{−/−} mice. The reasons for the ineffectiveness of human CRP in murine models of atherosclerosis are not defined. It is possible that an inflammatory environment, such as those characterized by acidic pH, is needed for efficient interaction between CRP and atherogenic LDL during the development of atherosclerosis and to observe the possible atheroprotective function of CRP in animal models.

Keywords

Atherosclerosis; C-reactive protein; Enzymatically-modified low-density lipoprotein; Foam cells; Oxidized low-density lipoprotein

INTRODUCTION

C-reactive protein (CRP), which is produced by hepatocytes and circulates in the plasma, is a pentameric protein comprised of five identical, non-covalently associated subunits [reviewed in 1–3]. The native structure of CRP is altered in response to a variety of *in vitro* experimental conditions and due to its localization at the sites of inflammation *in vivo*. For example, the conformation of the pentameric structure of CRP is altered at acidic pH [4–6, and our unpublished observations]. After binding to cell membranes and activated platelets, CRP dissociates into its individual subunits generating the monomeric form of CRP [7–11]. The monomeric form of CRP can be distinguished from pentameric CRP by using specific monoclonal antibodies. It is also important to recognize that when purified CRP is stored at high concentrations in calcium-containing buffers, some CRP molecules form decamers, oligomers and aggregates [12, 13]. There is always a possibility that the stored preparations

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of purified native pentameric CRP may contain some decamers and soluble aggregates of CRP and that the aggregated protein, when exposed to growing vascular cells in culture, may generate toxic environment in the cell culture medium. This possibility is usually considered when the experiments are planned to investigate the effects of native pentameric CRP on vascular cells.

The serum concentration of CRP increases in inflammatory disease and, therefore, CRP has been used as a marker of inflammation. Minor elevation of serum CRP levels is associated with increased risk of atherosclerosis and subsequent cardiovascular disease in general populations. However, the data to suggest an answer to the following two questions remain controversial [reviewed in 14–16]: 1. Is CRP an independent risk factor for atherosclerosis and predict the disease, and 2. Is CRP causally involved in the pathogenesis of atherosclerosis and should be regarded as a primary therapeutic target for prevention of the disease?

Atherosclerosis is caused by the retention and modification of low-density lipoprotein (LDL) in artery walls. Modified LDL is engulfed by macrophages to form foam cells that contribute to the development of atherosclerotic lesions [17]. CRP has been implicated in modulating the pathogenesis of atherosclerosis because CRP is localized in atherosclerotic lesions, binds to modified LDL, and prevents the formation of macrophage foam cells. Atherosclerosis is an inflammatory disease and it has been suggested that in areas in which inflammation takes place, the pH may decrease to acidic levels due to activated macrophages, hypoxia, lactate generation and proton generation [18–22]. Thus, two of the many characteristics of the atherosclerotic lesions are the presence of modified LDL and the presence of acidic pH. In this article, we discuss the contribution of these two characteristics of the developing atherosclerotic lesions to the functions of CRP in atherosclerosis.

LOCALIZATION OF CRP IN ATHEROSCLEROTIC LESIONS

In normal healthy individuals, the median concentration of CRP in the serum is 0.8 µg/ml; in acute phase, the concentration increases to 500 µg/ml or more [23]. Although the circulating concentration of CRP increases only minimally in atherosclerosis, CRP is localized in atherosclerotic lesions. Thus, the localization of CRP in atherosclerotic lesions is independent of the concentration of CRP in the circulation. CRP has been shown to be localized in atherosclerotic lesions of coronary arteries in both humans [24–30] and experimental animals [31–34]. CRP is also present in the myocardium of patients of myocardial infarction and dilated cardiomyopathy [35–37]. Likely, the diverse ligand-binding specificities of CRP, at physiological pH or acidic pH, are responsible for the localization of CRP in atherosclerotic lesions and infarcted myocardium.

Polyclonal antibodies to native pentameric CRP are generally used to detect the presence of CRP in atherosclerotic lesions. However, antibodies that do not react with native pentameric CRP but are specific for monomeric CRP have also been successfully used to detect the presence of CRP in atherosclerotic lesions. The detection of CRP by using monomeric CRP-specific monoclonal antibodies reflects the presence of monomeric CRP in atherosclerotic lesions [7, 9]. Monomeric CRP has also been seen in normal vascular tissue [38]. It is, however, not known whether the pentameric CRP localizes first and then, after localization, pentameric CRP is converted to monomeric CRP. It has been shown that the binding of CRP to liposomes and cell membranes leads to structural changes in pentameric CRP and that the altered pentameric CRP reacts with the antibodies specific for monomeric CRP [8]. The binding of CRP to activated platelets and apoptotic cells has also been shown to monomerize CRP [9, 10]. These findings indicate that monomeric CRP is a by-product of the binding of CRP to its ligands and explain the detection of CRP in atherosclerotic lesions

by using antibodies specific for monomeric CRP. The presence of monomeric CRP in atherosclerotic lesions also suggests that CRP was there to execute a ligand-binding function and that its function was complete. If the monomeric CRP in atherosclerotic lesions is always ligand-bound and not free, then the data obtained from the use of free monomeric CRP produced *in vitro* by various means is physiologically less relevant.

REQUIREMENTS FOR THE BINDING OF CRP TO LDL

The deposition of CRP in atherosclerotic lesions is justified by the known ligand-binding specificities of CRP. At physiological pH, CRP exhibits calcium-dependent binding to molecules and cells bearing exposed phosphocholine moiety, such as apoptotic cells and necrotic cells present in myocardial infarcts [39, 40].

The binding of CRP to modified forms of LDL depend on the pH. In *in vitro* experiments, two forms of modified LDL are used as models of atherogenic forms of LDL to investigate the functions of CRP in atherosclerosis. One form is called enzymatically-modified LDL (E-LDL) which is prepared by treating LDL with plasmin and cholesterol esterase under defined conditions [26]. The other form is called oxidized LDL (ox-LDL) which is prepared by treating LDL with copper chloride under defined conditions [40]. At physiological pH, CRP binds to E-LDL in a calcium-dependent manner [26, 41–43]. However, the binding of CRP to E-LDL is more efficient at acidic pH than at physiological pH [5]. It has been suggested that, at physiological pH, CRP binds to E-LDL through the phosphocholine groups present in E-LDL [26, 41–43]. Although the binding of CRP to E-LDL is mediated by the phosphocholine-binding site of CRP, the amino acids in CRP that contact phosphocholine are not critical for the binding of CRP to E-LDL [43]. Thus, the phosphocholine groups present in E-LDL are not the only components in E-LDL through which CRP binds to E-LDL. Importantly, it has been shown that CRP also binds to purified non-esterified cholesterol [41, 44]. The binding capability of CRP to ox-LDL at physiological pH is controversial. Several laboratories have reported that CRP binds to ox-LDL at physiological pH [40, 42, 45, 46]. It has also been indicated that CRP binds to ox-LDL *in vivo* in diabetes mellitus patients with atherosclerosis and in experimental animals [47, 48]. On the other hand, several investigators found that CRP did not bind to ox-LDL at physiological pH [41, 49, 50]. Acidic pH was required for the binding of CRP to ox-LDL [5].

CRP does not bind to native LDL [40, 41, 45, 51]. However, if either CRP or native LDL is immobilized and aggregated, then CRP and native LDL interact with each other [52–54]. Immobilized CRP also binds to very low-density lipoprotein [52]. The complexes of CRP and very low-density lipoprotein have also been identified in the serum [55, 56]. Recently, it has been shown that CRP also binds to LOX-1 which is a receptor for ox-LDL [57, 58]. We have shown that fluid-phase CRP binds to a variety of immobilized proteins if both CRP and the immobilized proteins are treated with acidic pH [5]. In this regard, CRP, at acidic pH, is similar to heat shock proteins which bind to all conformationally altered proteins. Interestingly, several polyclonal and monoclonal anti-CRP antibodies have been shown to cross-react with human heat shock protein Hsp60 [59]. The significance of such diverse binding specificities of CRP in atherosclerosis is unclear.

PREVENTION OF FOAM CELL FORMATION

The formation of modified LDL-loaded macrophage foam cells represents an early step in atherosclerosis. The formation of foam cells begins when macrophages bind and take up modified LDL. Components in modified LDL including cholesteryl ester are hydrolyzed in lysosomes. The resulting free cholesterol is transferred to the endoplasmic reticulum and then re-esterified to cholesteryl esters, which accumulates in the cytosol to form intracellular

lipid droplets. Intracellular accumulation of lipid droplets results in the formation of foam cells [reviewed in 60].

The effects of CRP on the uptake of modified LDL by macrophages and on the formation of foam cells have been investigated from time to time for last 15 years. Hatanaka *et al.* hypothesized that CRP may facilitate the uptake of lipids by macrophages in atherosclerotic lesions [25]. In this study, using immunohistochemical staining with anti-CRP antibodies, CRP was located intracellularly in foam cells, suggesting uptake of CRP-lipid complexes by macrophages [25]. Zwaka *et al.* concluded that the formation of foam cells might be caused in part by uptake of CRP-opsonized native LDL [61]. In this study, a mixture of CRP and native LDL was used for the treatment of macrophages to assess the effects of CRP on the uptake of native LDL by macrophages. Native LDL incubated with CRP was taken up by macrophages as determined by immunofluorescent labeling and microscopy. Thus, in this study, CRP bound to native LDL in the fluid phase and the complexes were taken up by macrophages [61]. Fu *et al.* reported that CRP might contribute to the formation of foam cells by causing aggregation of native LDL [54]. In this study, CRP was aggregated by immobilization of CRP to microtiter wells. Native LDL was then captured on the wells through immobilized CRP. After the macrophages were added to the wells, the immobilized CRP-LDL complexes were somehow detached from the microtiter wells and taken up by the cells. Thus, in this study, assuming that CRP is deposited in the arteries prior to the retention of LDL and that the deposited CRP recruits LDL, the effect of CRP seems to be pro-atherosclerotic [54]. Verma *et al.* reported that CRP increased the uptake of native LDL by macrophages [62]. In this study, the effect of CRP on the uptake of LDL was assessed indirectly by measuring the internalization of CD32, a receptor to which free CRP was supposed to bind [62]. Van Tits *et al.* reported that CRP enhances the binding of ox-LDL to macrophages [45]. Singh *et al.* reported that CRP promoted ox-LDL uptake by macrophages both *in vitro* and *in vivo* [48]. In this study, CRP also increased intracellular cholesteryl ester accumulation in macrophages [48]. Schwedler *et al.* reported that CRP did not decrease the uptake of acetylated LDL by endothelial cells [63]. In all these studies, the effect of CRP on the uptake of LDL by macrophages was assessed mostly by using mixtures of CRP and LDL for the *in vitro* treatment of macrophages, and a possible function of CRP in preventing the formation of foam cells was not revealed.

We recently investigated the effects of CRP on the accumulation of lipid droplets made up of cholesteryl esters in E-LDL-treated macrophages [43]. We isolated the CRP-E-LDL complexes from the mixture of CRP and E-LDL and used these isolated complexes to treat macrophages. We found that, in contrast to E-LDL alone, CRP-bound E-LDL was inactive in the formation of foam cells, suggesting that CRP could prevent the formation of foam cells. These findings were similar to those of Mookerjee *et al.* who reported that the complexes of CRP and LDL were unable to enter macrophages [64]. CRP, after binding to LDL, caused charge modification of LDL, and the degradation of modified LDL by macrophages was increased in the presence of CRP [64]. Collectively, these reports raised the possibility that if CRP was present in sufficient amount in the arterial wall, if each LDL molecule retained in the arterial wall became CRP-bound, and if the conditions were suitable for CRP to bind to modified LDL, then CRP might be capable of preventing foam cell formation *in vivo*.

INEFFECTIVENESS OF CRP IN ANIMAL MODELS OF ATHEROSCLEROSIS

To determine the role of CRP in the development of atherosclerosis, human CRP has been used in two different murine models of atherosclerosis, *ApoE*^{-/-} mice and *Apob*^{100/100}*Ldlr*^{-/-} mice. In *ApoE*^{-/-} mice, in most studies, CRP was found to be neither pro-atherogenic nor atheroprotective [32, 65–70]. Both passively administered human CRP

and transgenically expressed CRP had no effect on the development, progression, or severity of spontaneous atherosclerosis in mice. In *Apob*^{100/100}*Ldlr*^{-/-} mice, which are rich in LDL and develop human-like hypercholesterolemia, CRP slowed the development of atherosclerosis [71]. However, in another study using the same mice, there was no effect of CRP on the development of atherosclerosis [72]. The functions of CRP have also been investigated in a rabbit model of atherosclerosis. Human CRP did not affect aortic or coronary atherosclerosis lesion formation in rabbits transgenic for human CRP [34].

Thus, the *in vitro* data on the binding of CRP to modified LDL and on the effects of CRP on the formation of foam cells implicating CRP as an atheroprotective molecule could not be extended to *in vivo* situations. The *in vitro* data suggested that CRP should be able to bind to modified forms of LDL *in vivo* due to localized decrease in extracellular pH in atherosclerotic lesions. The reasons for the ineffectiveness of CRP in murine models of atherosclerosis are not defined. It is known that atherosclerosis is not naturally developed in mouse models as it is developed over a period of several years in humans [73]. It is possible that an inflammatory environment, such as those characterized by acidic pH, which can alter the pentameric structure of CRP, is what may be needed for an efficient interaction between CRP and atherogenic LDL during the development of atherosclerosis and to observe the possible atheroprotective function of CRP in animal models.

CONCLUSIONS AND FUTURE PERSPECTIVES

In vitro data suggesting that CRP should function as an atheroprotective molecule have not yet been confirmed in animal models of atherosclerosis. Let us consider that the LDL-binding function of CRP in the development of atherosclerosis is not mediated by CRP in its native pentameric structure but by CRP in its inflammation-dependent pentameric structure. We then need to generate CRP mutants, by site-directed mutagenesis, which are efficient in binding to modified LDL at physiological pH. These CRP mutants then can be evaluated for their effects on the development of atherosclerosis in available animal models.

For this article, we did not review the other set of data revealing the effects of CRP on vascular cells. The described effects of CRP on vascular cells indicate a pro-inflammatory role of CRP in atherosclerosis [74–77]. It has been demonstrated that the pro-inflammatory action of CRP on vascular cells in culture are solely the effects of CRP and not of sodium azide and endotoxins that might have been present in some CRP preparations [78–83]. Notably, in control experiments using albumin which, unlike CRP, does not have a tendency to self-aggregate upon storage, albumin had no pro-inflammatory effects on cultured vascular cells. This aspect of atherosclerosis-related functions of CRP has been reviewed elsewhere [76, 84, 85].

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ABBREVIATIONS

CRP	C-reactive protein
LDL	Low-density lipoprotein
E-LDL	Enzymatically-modified LDL
Ox-LDL	Oxidized LDL

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