Effect of Modified C-Reactive Protein on Complement Activation

A Possible Complement Regulatory Role of Modified or Monomeric C-Reactive Protein in Atherosclerotic Lesions

Shang-Rong Ji, Yi Wu, Lawrence A. Potempa, Yu-Heng Liang, Jing Zhao

Objective—The capacity of human C-reactive protein (CRP) to activate/regulate complement may be an important characteristic that links CRP and inflammation with atherosclerosis. Recent advances suggest that in addition to classical pentameric CRP, a conformationally distinct isoform of CRP, termed modified or monomeric CRP (mCRP), may also play an active role in atherosclerosis. Although the complement activation behavior of CRP has been well established, the capacity of mCRP to interact with and activate the complement cascade is unknown.

Methods and Results—mCRP bound avidly to purified C1q, and this binding occurred primarily through collagen-like region of C1q. Fluid phase mCRP inhibited the activation of complement cascade via engaging C1q from binding with other complement activators. In contrast, when immobilized or bound to oxidized or enzymatically modified low-density lipoprotein, mCRP could activate classical complement pathway. Low-level generation of sC5b-9 indicated that the activation largely bypassed the terminal sequence of complement, which appears to involve recruitment of Factor H.

Conclusions—These results indicate that mCRP can both inhibit and activate the classical complement pathway by binding C1q, depending on whether it is in fluid phase or surface-bound state. (Arterioscler Thromb Vasc Biol. 2006;26:935-941.)

Key Words: modified C-reactive protein • complement • low-density lipoprotein

Several lines of evidence demonstrate the involvement of the complement cascade in atherogenesis. Immunohistochemical staining revealed the presence of complement components1–3 in both early and advance human atherosclerotic lesions, indicating that complement activation occurred at an early stage in atherosclerosis. In addition, RNA analyses have indicated that complement genes are expressed locally within plaques.4 A proatherosclerotic role of terminal complement sequence was suggested because C65 and C36 deficiency in both normal arteries and atherosclerotic lesions,8,9 is of special interest. Although CRP had long been regarded only as a sensitive prognostic marker, evidence is now accumulating to suggest its direct participation in the atherosclerotic process.10,11 CRP is known to activate the classical complement pathway (CCP) when bound to multivalent ligands and is capable of regulating alternative complement pathway (ACP) in a mechanism involving recruiting Factor H.12 CRP can modulate complement activation properties of enzymatically modified LDL (E-LDL)13 and colocalize with activated complement components6 and various LDL derivatives such as oxidized LDL (ox-LDL) and E-LDL13–15 in human atherosclerotic lesions. The capacity of CRP to bind LDL and activate/modulate the complement cascade suggests that CRP may play an important role in regulation of complement activation during atherosclerosis process. Recent advances16,17 indicate that transgenic CRP has no effect on atherosclerosis development in apoE−/− mice and propose that this animal model is not adequate for exploring the atherosclerotic effect of CRP, which may be partly related to the oddities of the mouse complement system.17

CRP is composed of 5 identical globular subunits arranged as a cyclic pentamer. When the subunits are separated into monomers, they undergo a conformational change that sig-
significantly modifies solubility and antigenicity and presents very distinct biological activities.\textsuperscript{18–25} This alternative form of CRP is termed modified or monomeric CRP (mCRP). An mCRP antigen is preferentially expressed in tissues\textsuperscript{18,26} in contrast to CRP, which is a serum-based protein. mCRP is a predominant antigen detected in human arterial intima.\textsuperscript{26} In addition, although the expression of CRP mRNA in both normal and plaque arterial tissues has been identified,\textsuperscript{4,27,28} it remains to be proven whether CRP or mCRP isoform is finally produced by extracellular tissues. In fact, recent evidence indicates that CRP mRNA is processed into mCRP normally in exist in $\geq 2$ isomeric forms and that each has distinct bioactivities, studies that clearly define and differentiate how each isoform may participate in underlying process of atherosclerosis are needed.

The capacity to activate/regulate complement is an important characteristic that links CRP with inflammation and with the suggested role of inflammation in atherosclerosis. Because the monoclonal antibody (mAb; Clone CRP-8) used in several studies\textsuperscript{2,13–15} to illustrate colocalization of CRP with complement predominantly recognizes mCRP, we investigated the direct capacity of mCRP to regulate the complement system in the present work.

Materials and Methods
CRP/mCRP batches were dialyzed to remove NaN$_3$ and assayed for endotoxin contaminant by Limulus assay (Sigma) before use. The final endotoxin level of all protein solutions was <0.06 EU/mL. For details on protein preparation, please refer to the online supplemental Materials and Methods, available at http://atvb.ahajournals.org.

Fluid Phase Binding of mCRP to Immobilized C1q
A total of 5 $\mu$L C1q (5 $\mu$g/mL) in coating buffer (10 mmol/L sodium bicarbonate, pH 9.6) was incubated in microtiter wells at 37°C for 2 hours. The wells were washed 3 times with TBS (10 mmol/L Tris, 140 mmol/L NaCl, pH 7.4) containing 0.02% Nonidet P-40 for 5 minutes and blocked with 1% BSA/TBS for 1 hour at 37°C. Biotinylated mCRP or CRP in 1% BSA/TBS containing 0.06 EU/mL. For detection of iC3b and sC5b-9 was measured by commercial ELISA kits (Quidel).

Other Complement Assays
To examine C3a activation, EA (antibody sensitized sheep erythrocytes) hemolytic activity assays were performed. Various amounts of mCRP, LDL, or mCRP–LDL complex were preincubated with 5% NHS diluted in VBS$_{Ca}$ for 1 hour at 37°C before addition of 1 x $10^9$ EA in VBS$_{Ca}$. After 30 minutes of incubation at 37°C, VBS$_{EDTA}$ was added to stop the reaction. After centrifugation, released hemoglobin was quantified at an OD of 412 nm. mCRP–LDL complex was produced by coincubation of mCRP and LDL in VBS$_{Ca}$ (VBS containing 0.15 mmol/L calcium) for 1 hour at 37°C. To examine C5a activation, RRBC (rabbit red blood cell) hemolytic activity assays were performed. Various amounts of CRP or mCRP were preincubated with 50% NHS diluted in VBS$_{Ca}$ for 1 hour at 37°C and then added to 2 x $10^9$ RRBCs in VBS$_{Ca}$ for 30 minutes. The reaction was stopped by addition of VBS$_{EDTA}$ and 412-nm OD was measured.

For detection of inactivated C3b (iC3b) and soluble C5b–C9 complexes (sC5b-9), mCRP, LDL, or mCRP–LDL complex was preincubated with 5% NHS, and then iC3b and sC5b-9 were measured by commercial ELISA kits (Quidel).

Statistical Analysis
All measurements were performed in duplicate, and each experiment was repeated 3 to 5 times. Data were compared between experimental groups by 1-way ANOVA followed by the Bonferroni multiple range test using OriginPro 7.5 software (OriginLab Cooperation). The normality of data were verified by Shapiro–Wilks normality test. A value of $P<0.05$ was considered significant. Data are presented as mean±SEM.

Results
Binding of mCRP to C1q
We first investigated the interactions between mCRP and C1q, the initial component of the CCP. Microtiter wells were coated with fixed amount of C1q and assessed for binding of biotinylated mCRP (Figure 1A); alternatively, wells were coated with mCRP, and C1q binding was detected (Figure 1B). The results indicated that mCRP, either in immobilized phase or fluid phase, bound C1q avidly in a dose-dependent fashion. As negative control, no binding of C1q to BSA was observed.

In contrast to mCRP, CRP exhibited rather weak binding to C1q and was transformed to a potent ligand for C1q binding after cross-linking (ie, Bis-aggCRP; Figure 2A), in agreement with Jiang et al.\textsuperscript{30} A recent study reported that after immobilized onto microtiter wells, rabbit CRP bound C1q strongly.\textsuperscript{31} Our results using CRP directly absorbed onto polystyrene (latex) microtiter plates also supported this observation (Figure 2B). However, further experiments indicated that substantially conformational change occurred in CRP after direct immobilization such that immobilized CRP predominantly expresses mCRP antigenicity (>95%) and is not recognized by mAbs against CRP determinants\textsuperscript{32} (Figure 2C). As positive controls, CRP retained pentameric CRP antigenicity by ELISA when captured as a function of calcium onto phospholipidine-conjugated BSA and by dot-blot assays...
when CRP is adsorbed onto nitrocellulose (an ionized surface; data not shown), consistent with previous reports.32,33 These results indicated that conversion from CRP to mCRP rendered this molecule more active in C1q binding and abolished the prerequisite of complexed with multivalent ligand or cross-linking.

Additional experiments were performed to locate the site of C1q involved in the interaction with mCRP. In solid phase C1q-binding assays, preincubation with agg-IgG (aggregated human IgG) did not inhibit the binding of biotinylated mCRP. Preincubation with mCRP (black bar) or agg-IgG (gray bar) to immobilized globular region and collagen-like region of C1q. A total of 5 μg/mL globular region or collagen-like region was immobilized in microtiter wells for 2 hours at 37°C followed by blocking. Then biotinylated mCRP or agg-IgG (5 μg/mL) was added and detected for protein binding. Each data point represents the mean of 3 independent experiments.
to immobilized C1q (Figure 3A). However, preincubation with mCRP partially inhibited binding of biotinylated agg-IgG to immobilized C1q. Because agg-IgG is known to bind globular region of C1q, the above results suggested mCRP might primarily interact with collagen-like region of C1q. To further address this point, we assayed the binding of mCRP to separated collagen-like region and globular region of C1q. As shown in Figure 3B, agg-IgG only bound to globular region, whereas mCRP bound both fragments, with predominant binding to collagen-like region.

**Fluid Phase mCRP Block Complement Activation Via Engaging C1q**

To examine whether mCRP–C1q binding can activate complement, hemolytical assays were performed. Preincubation of NHS with mCRP resulted in dose-dependent decrease in hemolytic activity of EA through CCP (Figure 4A). On the other hand, mCRP addition to NHS had only a marginal effect on hemolytic activity of RRBCs through ACP (Figure 4B). These results are consistent with the interpretation that mCRP can consume the complement components in NHS necessary for CCP processing but not for ACP processing. Because C3 is the converging point of both CCP and ACP, the effect of fluid mCRP on C3 activation was accessed by evaluating either the formation of mCRP–C3d complex or the generation of iC3b. Comparably generation of iC3b was observed in both NHS and NHS preincubated with mCRP (Figure 4C), and only trace levels of mCRP–C3d complex could be detected (data not shown). This indicated that addition of mCRP in NHS did not result in C3 turnover, suggesting successive complement cascade was not activated by fluid phase mCRP–C1q binding. Rather, it is most likely that such binding engages C1q from interacting with other activators such as EA and hence blocks further activation of complement system. In line with this speculation, mCRP dose-dependently inhibits the binding of C1q to EA (Figure 4D). Together, these findings support the concept that the binding of free mCRP molecule to C1q does not activate complement, possibly caused by inefficiency in activating C1q hexamer. Similar fluid phase inhibition of complement has also been reported for PTX3, a prototypic long pentraxin.

**Immobilized mCRP Activates CCP**

Because fluid phase (or ligand free) mCRP could interact with and inhibit the hemolytic activity of the CCP on EA (Figure 4), further experiments were performed to identify whether mCRP immobilized on ELISA plates could similarly affect the complement system. Immobilized mCRP was incubated with serum, and C3d deposition was measured by ELISA. Addition of NHS led to C3 activation on coated mCRP but not on BSA (Figure 5A). When NHS was added in the presence of Mg-EGTA, C3d deposition was almost abolished, indicating the effect of mCRP on C3d deposition predominantly involves CCP. Further support was provided by the experiments using C1q-deficient human serum (C1q-D-HS) as the complement source, which resulted in no deposition of C3d on immobilized mCRP (Figure 5B). Addition of purified C1q to the C1q-D-HS could restore C3d deposition. As shown in Figure 2 and in a previous report, immobilized CRP exclusively exhibited mCRP antigenicity, thus the complement activation of immobilized CRP was tested. Similar to immobilized mCRP, immobilized CRP also exclusively activated CCP, which was in line with the previous report using rabbit CRP in solid phase complement activation assays.31

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**Figure 4.** A, Inhibition of CCP EA hemolytic activity by CRP (贽) or mCRP (ŋ). Various amounts of CRP or mCRP were preincubated with 5% NHS diluted in VBS\(^{Ca}\)\(^{+}\)\(Mg\) for 1 hour at 37°C and were then added to 1×10\(^6\) EA in VBS\(^{Ca}\)\(^{+}\)\(Mg\). After 30 minutes of incubation at 37°C, VBS\(^{EDTA}\) was added to stop the reaction. After centrifugation, 412-nm OD of the reaction mixture was measured. *P<0.05; **P<0.01 against of EA inhibition by CRP at equal concentration. B, Inhibition of ACP RRBC hemolytic activity by CRP (贽) or mCRP (ŋ). Various amounts of CRP or mCRP were preincubated with 50% NHS diluted in VBS\(^{Ca}\)\(^{+}\)\(Mg\) for 1 hour at 37°C and were then added to 2×10\(^7\) RRBCs in VBS\(^{Mg}\)\(^{+}\)\(EGTA\) for 30 minutes. The reaction was stopped and 412-nm OD was detected. C, Various amounts of mCRP (ŋ) or agg-IgG (贽) was preincubated with 5% NHS for 1 hour, and generation of iC3b was determined by an ELISA kit. *P<0.05; **P<0.01 against iC3b generation by mCRP. D, mCRP inhibits binding of C1q to EA. EA was incubated with 5% NHS in the presence of various concentrations of mCRP at 4°C. C1q binding was accessed by goat-anti human C1q and fluoresceinisothiocyanate-conjugated rabbit anti-goat antibody (Table I, available online at http://atvb.ahajournals.org). Similar competition results were also obtained by using EA and isolated C1q (instead of NHS). *P<0.05; **P<0.01 against residue binding of C1q on EA with NHS alone. Each data point represents the mean of 5 independent experiments.
mCRP Regulate Complement Activation Behavior of Modified LDL

Native LDL (n-LDL) modified by oxidation or enzyme are both of atherosclerotic relevance and are shown to be colocalized with CRP (stained with CRP-8 Clone from Sigma) in human atherosclerotic lesions. Because mCRP could avidly bind to these LDL derivatives (Figure I, available online at http://atvb.ahajournals.org), we examined the effect of mCRP-bound LDLs on C1q binding and complement activation. As shown in Figure 6A, n-LDL and E-LDL did not bind C1q, whereas ox-LDL bound an appreciable amount of C1q. However, when coated with mCRP, all 3 LDL derivatives showed substantial binding to C1q (Figure 6A). Complement activation of the different mCRP–LDL complexes was further investigated by measuring EA hemolytic activity and generation of activated complement products. At a concentration of 5 μg/mL, neither mCRP alone (Figure 4A) nor LDL derivatives alone (Figure 6B) significantly inhibited NHS hemolytic activity on EA. However, addition of LDL derivatives preincubated with 5 μg/mL mCRP did significantly inhibit NHS CCP hemolysis (Figure 6B).

Unlike the absence of C3 activation by fluid phase (free) mCRP, ox-LDL or E-LDL complexed with mCRP, but not n-LDL complexed with mCRP, induced obvious consumption of C3 (Figure 6C), indicating progression of early complement cascade. The exclusive CCP activation of mCRP–LDL complex was demonstrated by lack of iC3b generation when C1qD-HS was used as complement source (data not shown). Further analysis showed that C3 activation was not paralleled by generation of terminal sequence product, only a small increase in sC5b-9 generation could be noted compared with baseline levels. Thus, complement activation by mCRP–LDL complex does not involve the more inflammatory and destructive terminal sequence. Control experiments indicated that coinubation of CRP with n-LDL or ox-LDL did not induce complement consumption, whereas CRP–E-LDL complex showed enhanced complement activation (data not shown). These results were in line with the observation of Bhakdi et al. Mold et al reported that the regulatory role of CRP on complement activation was mediated by Factor H binding. They reported only immobilized CRP could bind Factor H and thus proposed that the immobilization exposed additional sites on CRP for Factor H binding. As depicted in Figure 2 and previous reports, immobilized CRP actually expressed

Figure 5. A, C3d deposition on immobilized mCRP (white bar), CRP (black bar), or BSA (gray bar). A total of 5 μg/mL proteins was immobilized overnight at 4°C. After blocking with 1% BSA, 1% NHS diluted in VBS, or VBS/EGTA, or VBS/EDTA was added in protein-coated wells at 37°C for 1 hour. C3d deposition was detected by sheep anti-human C3d polyclonal IgG. *P<0.01 against C3d deposition in NHS-EDTA. B, 1% NHS, C1qD-HS, or C1qD-BS supplemented with purified C1q (2 μg/mL) in VBS/EDTA was added to mCRP-coated wells, and C3d deposition was measured. Each data point represents the mean of 3 independent experiments.

Figure 6. A, Binding of C1q to immobilized LDL (white bar) or mCRP-coated LDL (black bar). A total of 10 μg/mL LDL was immobilized onto microtiter wells overnight at 4°C. Then 5 μg/mL mCRP was added for 1 hour at 37°C. After washing, 5 μg/mL C1q was added for another 1 hour, and then the binding was detected as in Figure 1. *P<0.05; **P<0.01 against C1q binding to LDL alone. B, Inhibition of EA hemolytic activity by LDL alone (white bar) or mCRP–LDL complex (black bar). A total of 5 μg/mL LDL with or without 1 hour preincubation with 5 μg/mL mCRP was added to 5% NHS diluted in VBS for 1 hour at 37°C and then assayed for EA hemolysis as in Figure 4A. *P<0.05; **P<0.01 against EA inhibition by LDL alone. C, A total of 5 μg/mL LDL with or without 1-hour preincubation with 5 μg/mL mCRP was reacted with 5% NHS, and iC3b and sC5b-9 generation was determined by ELISA kits. *P<0.05; **P<0.01 against iC3b/sC5b-9 generation in serum alone. D, Various amount of Factor H was added to mCRP-coated wells, and the binding was detected with sheep anti-human Factor H IgG (Binding Site). Each data point represents the mean of 3 to 5 independent experiments.
mCRP antigenicity or, in other words, immobilized CRP present additional site(s) usually inaccessible in pentameric structure but exposed in monomeric structure. This prompted us to examine the interaction between mCRP and Factor H. As shown in Figure 6D, dose-dependent Factor H binding to immobilized mCRP could be observed.

Discussion

CRP is a serum protein mainly found in the circulation that is widely appreciated by the scientific and medical communities as the most reliable diagnostic marker for inflammation. However, although it is linked as a marker for inflammation, significant accumulation of CRP in inflammatory or injured tissues has not been noted, and the role it plays as a stimulator or inhibitor of inflammatory processes continues to be an enigma. In light of the new and evolving appreciation that CRP can exist in ≥2 conformationally distinct isoforms (ie, native CRP and mCRP; for a detailed discussion about the mechanism of mCRP production please refer to the online supplement), studies are needed to specifically compare and contrast the bioactivities of each conformer. Complement activation is considered to be an important source of inflammation in atherosclerosis, and CRP, because of its known capacity to activate complement and its colocalization to tissues containing atherosclerotic plaques, has been implicated in the disease process. In several of these studies, the mAb (Clone CRP-8) used to illustrate colocalization of CRP with complement predominantly recognizes mCRP. Hence, we sought to directly explore whether mCRP could also participate in complement activation. We clearly show that mCRP has the potential to interact with complement by binding to C1q and may regulate complement activation via 2 pathways, depending on whether mCRP is in fluid phase or bound to a surface. When mCRP is in ligand-free state, it exhibits complement inhibitory activity, presumably by restricting the binding of C1q with other complement activators. Because the activation of C1q needs ≥2 of its subunits to be closely engaged, this may account for the inability of fluid phase mCRP to further activate complement cascade after binding to C1q. When mCRP is surface immobilized either alone or bound to ox-LDL or E-LDL, it exhibits complement-activating activity of the early components of the CCP by binding to C1q, leading to the turnover of C3, largely bypassing the more inflammatory and destructive terminal sequence by recruitment of Factor H.

Together, our observations suggest a dual role for mCRP in the innate immune system. On the one hand, mCRP may facilitate the safe clearance of damaged self-materials that mCRP can bind (eg, modified LDL). On the other hand, mCRP can protect against unwanted complement activation in the fluid phase. It may be inferred from the present results that mCRP may have a protective role against complement-mediated inflammation in early stages of atherosclerosis. Unbound mCRP can inhibit complement activation by restricting the binding of C1q to CCP activators (eg, antibody-coated ox-LDL) and therefore negatively regulate the local production of inflammatory molecules generated by complement activation. On the other hand, the binding of mCRP to atherogenic LDL derivatives transform these particles into CCP activators. Because the complement activation by LDL-bound mCRP is largely restricted to early cascade, bypassing the terminal sequence, opsonic effect may play a major role in this pathway to facilitate the clearance of these potentially proatherosclerotic particles, as suggested by Bhakdi et al, concerning the interaction of CRP with E-LDL and complement. However, with the progression of atherosclerosis, the above suggested protective effect of mCRP on complement mediated inflammation may be counteracted by other factors such as localized accumulation of modified LDLS, increased damage to vascular tissues, exposure of subunal proteins, and activation of neutrophils and endothelial cells.

Acknowledgments

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Supplemental Materials and Methods

**CRP and mCRP**—CRP was either purified from human serum (>99% pure) or purchased from the Binding Site (>97% pure) (San Diego, CA) and Calbiochem (>99% pure, recombinant) (La Jolla, CA). The purchased CRP was further purified by anion-exchange chromatography\(^1\). All CRP preparations were stored in TBS\(^++\) (10mM Tris, 140mM NaCl, 2mM CaCl\(_2\), pH 7.4) to prevent spontaneous dissociation\(^2,3\). The functional integrity of CRP was verified by calcium dependent binding to PC-BSA (Biosearch Technologies, Novato, CA). The three sources of CRP exhibited similar activities.

Bis-aggCRP was prepared by cross-linking using bis (sulfosuccinimidyl) suberate (Pierce, Rockford, IL) according to manufacturer’s instruction, and separated by size-exclusion chromatography (SEC). CRP aggregates by this cross-linking can transform CRP to be an potent complement activator\(^4\). Recombinant mCRP\(^3\) was prepared as described. To simplify the experimental procedure, biotinylated CRP isoforms were mainly used. Biotinylation of proteins was performed according to manufacturer’s instruction (Pierce, Rockford, IL). The biotinylated proteins showed similar antigenicity and binding behavior comparing with their respective counterparts as reported.

Protein batches were dialyzed to remove NaN\(_3\) and assayed for endotoxin contaminant by Limulus assay (Sigma) before use. Additional purification step through Detoxi-Gel Columns (Pierce) was performed to remove endotoxin when necessary. The final endotoxin level of all protein solutions was below 0.06 EU/ml. Control experiments using 0.02ng/ml LPS (~3 fold higher than the maximal level of LPS that may exit in our protein solutions) were performed and no effect was observed in binding and complement assays.

**LDL and C1q**—Native LDL (n-LDL) was isolated from human plasma as described\(^5\), or purchased from Sigma (St. Louis, MO). LDL concentration was expressed in terms of protein. The purified and purchased n-LDL exhibited similar behavior in the present study. Oxidative and enzymatic modification and re-separation of LDL was performed as described\(^6\). All LDL preparations were used within one week. C1q (>95% pure) was obtained from Calbiochem. C1q collagen-like region and globular region were prepared by collagenase and pepsin digestion followed by SEC purification\(^4\). Aggregated IgG (agg-IgG) were produced by incubation of human IgG at 63°C for 45min. Biotinylation of agg-IgG was performed according to manufacturer’s instruction (Pierce, Rockford, IL). Factor H was purchase from Sigma.

**Inhibition of C1q binding to EA by mCRP**—1×10\(^8\) EA was incubated with 5% NHS in the presence of various concentration of mCRP. The incubation was performed at 4°C to minimize the activation of complement cascade, and thus avoid possible interference against detection of C1q binding. After 1h incubation, EA were separated, washed, followed by staining with goat-anti human C1q (10μg/ml) and FITC-conjugated rabbit anti-goat antibody (10μg/ml). Finally, EA was lysed by deionized water, and relative fluorescence intensity (RFI) was measured. The binding of C1q to EA was demonstrated by relative fluorescence intensity normalized with OD 412 nm (i.e. RFI/OD412nm).
Supplemental Discussion

Currently the origin of \textit{in vivo} mCRP remains largely unknown. We suggest that there may be two distinct but inter-complemental mechanisms for the \textit{in vivo} production of mCRP:

(i) \textit{Local expression}. A number of studies have identified CRP mRNA in extrahepatic cells such as human adipose tissue and smooth muscle cells in both normal and plaque arterial tissues\textsuperscript{7-9}. These reports, however, only describe a tissue-associated CRP antigen as the product of CRP mRNA translation. It remains to be proven whether the CRP subunit that is directly synthesized by extrahepatic cells is actually processed into the soluble, cyclic pentamer, or whether it is expressed as the mCRP isoform. Considering the wide tissue distribution of mCRP antigenicity\textsuperscript{10}, the possibility of local expression of mCRP can not be excluded. Indeed, a recent study\textsuperscript{11} revealed the generation of CRP by U937 macrophages after LPS stimulation, and suggested that cellular CRP is directly translated as the mCRP rather than the native CRP conformer. Thus, mCRP may be generated locally by extrahepatic cells when challenged by stimuli.

(ii) \textit{Local dissociation}. CRP can deposit into (inflammatory) tissues from circulation or can be directly generated by local vascular cells. Distinct forms of CRP including mCRP can be created in succession from pentameric CRP at the inflammation sites due to the local environmental change such as low pH, oxygen radicals, enzymatic actions etc.. In a \textsuperscript{125}I labeled CRP/mCRP injection study, local conversion from CRP to mCRP was suggested\textsuperscript{12}. Particularly, several investigations have shown that physiologically low pH (4.5–5.5)\textsuperscript{13}, oxygen radicals\textsuperscript{14}, or complexed with membrane\textsuperscript{15} could all promote the dissociation of CRP. These conditions may appear during atherosclerotic process, which is now regarded as a chronic inflammatory disease. The dissociation of CRP on membrane presumably produces surface bound mCRP, which may act in concert with CRP to facilitate complement dependent clearance of self-material (such as cell debris, apoptotic cell) or pathogens. The dissociation of CRP in response to radicals or low pH is likely to produce fluid-phase mCRP, which can inhibit complement activation and restrict the exacerbation of damage.
Table I mCRP inhibit binding of C1q to EA. $1 \times 10^8$ EA was incubated with 5% NHS in the presence of various concentration of mCRP. The incubation was performed at 4°C to minimize the activation of complement cascade, and thus avoid possible interference against detection of C1q binding. After 1h incubation, EA were separated, washed, followed by staining with goat-anti human C1q (10µg/ml) and FITC-conjugated rabbit anti-goat antibody (10µg/ml). Finally, EA was lysed by deionized water, and relative fluorescence intensity (RFI) was measured. Concurrently the sample was diluted 10 fold for OD412nm detection. The binding of C1q to EA was demonstrated by relative fluorescence intensity normalized by OD412nm.

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Supplemental Figures

Supplemental Fig.II Binding of mCRP to immobilized n-LDL, ox-LDL or E-LDL. Microtitre wells were coated with native or modified LDL (10µg/ml) over night at 4°C. After blocking, 100µl mCRP (5µg/ml) were added. mCRP binding were detected by 10µg/ml polyclonal goat anti-human CRP antibody (Sigma). Preliminary experiments indicated that this antibody recognized mCRP. mCRP-LDL binding was also tested by using other polyclonal CRP antibody (Binding Site) or biotinylated mCRP and HRP-conjugated avidin. All these assays produced similar results.

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