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Short communication

A STUDY OF THE INTERACTION OF THE C-REACTIVE PROTEIN MONOMER WITH THE U937 MONOCYTE

JING ZHAO[1] and XIN-HE SHI[2,*]
[1]The School of Life Sciences and [2]The Second Hospital of Lanzhou University, Lanzhou 730000, P. R. China

Abstract: C-reactive protein (CRP) has two structurally distinct isoforms, the CRP pentamer and the CRP monomer. A role for the CRP monomer in atherosclerosis is emerging, but the underlying mechanisms are only beginning to be understood. Monocytes are an important contributor to atherosclerosis, and foam cell formation is the hallmark of atherogenesis. However, whether the CRP monomer can directly interact with the monocytes and modulate their responses remains unknown. Furthermore, although FcγRIII (CD16) has been identified as the receptor for the CRP monomer on neutrophils, its role in mediating the CRP monomer's biological effects in other cell types has been questioned. In this study, we investigated the interaction of the CRP monomer with the monocytes using the U937 monocytic cell line. The CRP monomer specifically binds to U937 cells. This binding is unique in that it is independent of FcγRs and insensitive to protease digestion of the cell surface proteins. Further assays revealed that the CRP monomer directly incorporates into the plasma membrane. Interestingly, the presence of the CRP monomer efficiently retards oxidized low-density lipoprotein-induced foam cell formation of PMA-differentiated U937 macrophages and peripheral blood monocytic cell-derived macrophages. These findings provide additional evidence for the notion that the CRP monomer is an active CRP isoform that plays a role in atherogenesis via the direct modulation of the behavior of the monocytes.

Key words: C-reactive protein, Monocyte, Low-density lipoprotein, Foam cell

* Author for correspondence. e-mail: xinheshi.lzu@gmail.com, tel./fax: 86-931 -8942893

Abbreviations used: CRP – C-reactive protein; FcγR – Fc gamma receptor; LDL – low-density lipoprotein; ox-LDL – oxidized low-density lipoprotein; PBMC – peripheral blood monocytic cell; TBARS – thiobarbituric acid reactive substances

INTRODUCTION

Human C-reactive protein (CRP) is a typical acute phase protein composed of five identical subunits [1]. Increasing evidence indicates that in addition to the classical pentamer isoform, CRP can also exist as a monomer [2, 3]. These two isoforms exhibit distinct conformations, bioactivities and *in vivo* distribution patterns [2, 3]. It has emerged that both the CRP monomer [2, 3] and the CRP pentamer [4, 5] play roles in the development of atherosclerosis. It has been proposed that the tightly regulated interplay between these two isoforms endows CRP with its competence as a fine modulator of inflammation [6].

The CRP monomer has only recently been recognized as a naturally occurring CRP isoform, after considerable controversy, so the mechanisms whereby it participates in the underlying process of cardiovascular disease are just beginning to be understood. The CRP monomer may promote the endothelial dysfunction associated with the initiation and progression of atherosclerosis by inducing pro-inflammatory responses in endothelial cells [7]. Moreover, the CRP monomer may play a role in acute events of atherosclerosis through its capability to enhance platelet aggregation [8] and neutrophil activation [9-11].

Monocytes are an important contributor to the development of atherosclerosis [12], but whether the CRP monomer can directly interact with monocytes and modulate their responses remains unclear. Furthermore, although FcγRIII (CD16) has been identified as the receptor for the CRP monomer on neutrophils [13], this receptor appears not to be the major mediator of the CRP monomer's actions on endothelial cells [7] and platelets [8]. In this study, we investigated the interaction of the CRP monomer with the U937 monocytic cell line, finding evidence that the CRP monomer can directly regulate the behavior of monocytes in a CD16-independent manner.

MATERIALS AND METHODS

Reagents

CRP pentamer was purchased from Calbiochem. CRP monomer was generated from CRP pentamer via conventional urea-chelation [14]. The structural homogeneity of the CRP isoforms was verified via native electrophoresis and silver staining. FITC labeling was performed according to the manufacturer's instructions (Pierce). Native low-density lipoprotein (n-LDL) was purchased from Sigma. To prepare the oxidized LDL (ox-LDL), 1 mg/ml n-LDL was first dialyzed into EDTA-free buffer, and then dialyzed against 5 μM $CuSO_4$ at 37ºC for 18 h, followed by addition of 1 mM EDTA [15]. Heat aggregated IgG (HAG) was prepared by incubating 10 mg/ml human IgG at 63ºC for 60 min. The degree of LDL oxidation was assessed using the thiobarbituric acid reactive substances (TBARS) assay [16]. Heparan sulfate, chrondroitin sulfate A and chrondroitin sulfate B were purchased from Sigma. Anti-CD32 mAb, anti-CD16 mAb and isotype controls were purchased from BD Pharmingen.

Cellular binding

U937 monocytic cells were cultured in RPMI 1640 with 10% FBS, 5% CO_2. To evaluate binding of the CRP isoforms with U937 cells, 2×10^6 cells were incubated with different concentrations of FITC-labeled CRP pentamer or monomer in a binding buffer (1 mg/ml BSA and 10 mM NaN_3 in PBS with calcium and magnesium) for 30 min at 37ºC. The inclusion of NaN_3 efficiently blocked the cell internalization of the surface-bound FITC-labeled CRP monomer. Incubation with NaN_3 under these conditions did not result in appreciable changes in cell viability as evaluated by trypan blue staining. After binding, the cells were washed twice and transferred to a new tube to avoid the introduction of non-specific protein binding to the plastic tube wall during lysis. After two additional washes in the new tube, the cells were lysed by 0.1 M NaOH plus 0.1% SDS. The CRP isoform bound in ng was quantified by fluorescence intensity measurement according to a standard curve constructed using serial dilutions of the labeled protein. The total cellular protein was determined using BCA kits from Pierce. The final protein binding was expressed as ng CRP isoform bound/μg total cellular protein.

In the competition experiments, U937 cells were incubated with unlabeled CRP isoforms, 20 μg/ml of anti-FcγR mAbs or isotype IgG controls in the binding buffer for 30 min at 37ºC, followed by addition of 50 μg/ml of the FITC-labeled CRP monomer. In some experiments, 50 μg/ml of the FITC-CRP monomer was pre-incubated with 100 μg/ml of various glycosaminoglycan ligands for 30 min before being added to the cells. To remove the receptors on the cell surface, the U937 cells were treated with 15 μg/ml trypsin for 30 min before being used in the binding assays. In the post-binding treatments, after incubation with FITC-labeled CRP monomer, the cells were treated for 5 min with high salt (2 M NaCl), acid (50 mM glycine, pH 3.0) [17] or alkaline (pH 11.3) buffer [18], to evaluate the forces involved in CRP monomer-cell interaction. To determine the localization of the CRP monomer incorporated into the plasma membrane, post-binding extraction with 1% triton X-100 at 4ºC for 15 min was also conducted [18].

LDL uptake and foam cell formation

The differentiation of the U937 monocytes into macrophages was performed as described previously [19]. Rat PBMC were isolated, and non-adherent cells were removed after a 2-h culture. The adherent cells were cultured for 5 days to differentiate them into macrophages. The detached cells were seeded for 2 h at 37ºC (2×10^5 cells/well). After being washed, the cells were incubated with LDL or ox-LDL with or without the CRP monomer in RPMI 1640 at 37ºC, 5% CO_2 for 16 h. Foam cell formation was visualized by Nile red staining [20] and observed with fluorescence microscopy (Leica DMIL). In some experiments, the fluorescence intensity was quantified with a fluorescence spectrometer. 25 μg/ml polymyxin B was co-present during all of the experiments to neutralize the possible effects of LPS [21].

FcγR expression

The U937 expression of FcγR was evaluated via RT-PCR. The primers used are listed in Tab. 1.

Tab. 1. Primers for the amplification of FcγR genes.

Protein	Primer sequence	
	sense	anti-sense
CD64	5'-AGGTGTCATGCGTGGAAG-3'	5'-CCTGAGCAATGGTAGGTG-3'
CD32	5'-AGGCTGTGCTGAAACTCG-3'	5'-CGCTGTCATTGTTGTTGG-3'
CD16	5'-ACAAGCAGCAGGAAACATAGAAC-3'	5'-GATTACCATCCCTAGCCTGTATT-3'
GAPDH	5'-ACCACAGTCCATGCCATCAC-3'	5'-CCTGAGCAATGGTAGGTG-3'

Statistical analysis

All of the measurements were performed in duplicate and repeated 3-6 times. The data is shown as the means ± SE. Student's t-test and ANOVA were respectively used to assess the differences between two and more than two groups.

RESULTS

The positive expression of FcγRI (CD64) and FcγRII (CD32) in the U937 monocytes [22, 23] was confirmed via RT-PCR (Fig. 1A). These two receptors have been reported to mediate the cellular binding and biological responses of the CRP pentamer [23]. Accordingly, obvious binding of the CRP pentamer to U937 cells was observed (Fig. 1B) in agreement with the previous reports [23, 24]. By contrast, U937 does not express CD16 [22, 23], the putative receptor of the CRP monomer [13]. However, binding assays revealed strong binding of the CRP monomer to U937 cells with an apparent K_d of 88 ± 13 μg/ml (Fig. 1B). Pre-incubation with unlabeled CRP monomer efficiently suppressed the cellular binding of its labeled counterpart (Fig. 1C), indicating that the interaction of the CRP monomer with U937 monocytes is specific.

Additional experiments were performed to elucidate the nature of the interaction between the CRP monomer and U937 monocytes. Since FcγRs have been identified as the major receptors for the two CRP isoforms, we first tested the involvement of FcγRs using specific mAbs and a high affinity ligand (i.e. HAG) of FcγRs as the competitors. Although these reagents efficiently suppressed pentamer binding, only slight effects could be noticed on monomer binding (Fig. 2). Interestingly, removing the cell surface receptors via trypsin digestion merely resulted in a moderate decrease (~25%) in the level of CRP monomer binding (Fig. 2), suggesting the interaction of the CRP monomer and U937 monocytes is independent of protein receptors.

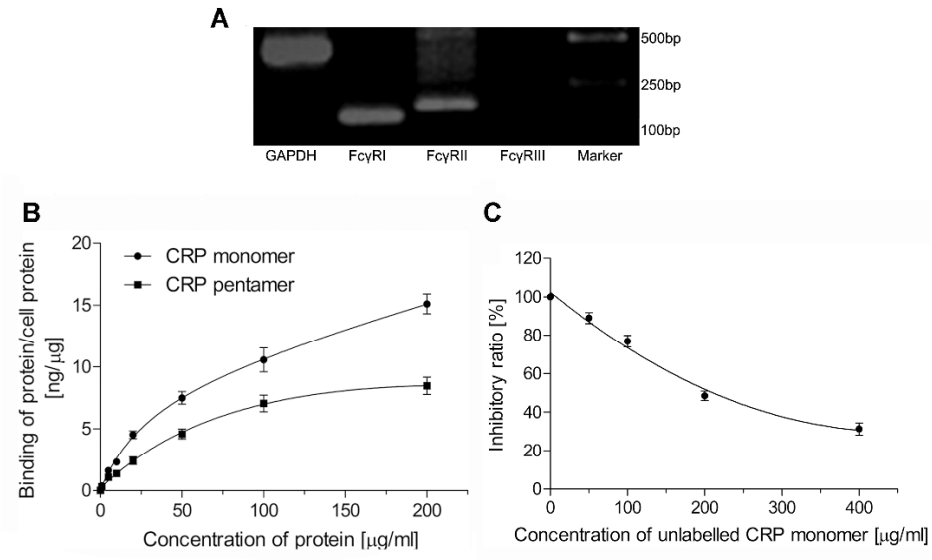

Fig. 1. The specific binding of the CRP monomer to U937 monocytes. A – U937 expression of FcγRI (143 bp), FcγRII (180 bp), FcγRIII (221 bp) and GAPDH (452 bp) by RT-PCR. B – Dose curves of FITC-labeled CRP pentamer and monomer binding to U937 monocytes. C – The competition of FITC-labeled CRP monomer binding with increasing concentrations of unlabeled CRP monomer.

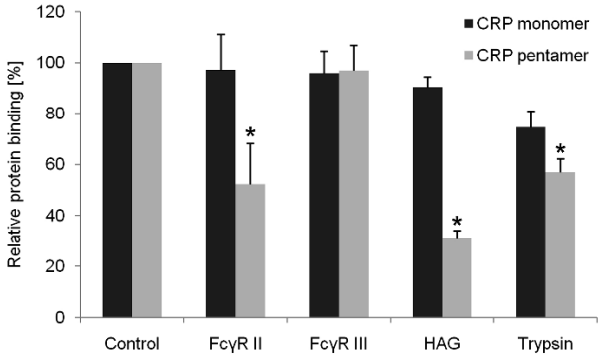

Fig. 2. The FcγRs-independent binding of the CRP monomer to U937 monocytes. U937 monocytes were pre-incubated with 20 μg/ml anti-FcγRII mAb, 20 μg/ml anti-FcγRIII mAb or 50 μg/ml HAG for 30 min, followed by the addition of 50 μg/ml FITC-labeled CRP monomer. The U937 monocytes were also pre-treated with 15 μg/ml trypsin for 30 min before the binding assays.

Glycosaminoglycan is a class of non-protein surface receptor, but several major cellular glycans, including heparan sulfate and chrondroitin sulfate A and B, failed to inhibit CRP monomer binding (Fig. 3A). To gain further insight into the forces underlying mCRP monomer interaction with U937 monocytes, cells with bound CRP monomer were treated with high levels of salt, acid or alkaline (Fig. 3B). High salt and acid rinses disrupt weak electrostatic interactions and strong

ligand-receptor binding, while an alkaline wash can reveal whether a protein is a membrane protein, because such harsh treatment will extract most of the peripheral proteins from the plasma membrane. However, these treatments yielded at best moderate inhibition, i.e. a ~35% decrease in CRP monomer binding due to the alkaline wash, suggesting that the cell-bound CRP monomer is incorporated into the plasma membrane. Cold triton extraction is routinely used to differentiate raft and non-raft resident membrane proteins. Interestingly, ~50% of the cell-associated CRP monomer, representing ~80% of the membrane-incorporated CRP monomer, is resistant to cold triton extraction. This indicates that membrane-inserted CRP monomer primarily locates in the cholesterol-enriched lipid raft microdomain.

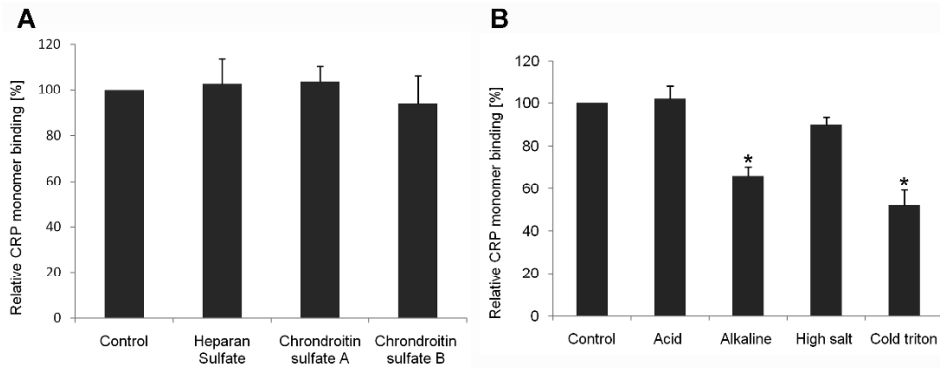

Fig. 3. The incorporation of the CRP monomer into the plasma membrane. A – U937 monocytes were pre-incubated with 100 μg/ml heparan sulfate, chrondroitin sulfate A or chrondroitin sulfate B for 30 min followed by the addition of 50 μg/ml FITC-labeled CRP monomer. B – After incubation with FITC-labeled CRP monomer, the cells were treated for 5 min with a high salt (2 M NaCl), acid (pH 3.0) or alkaline (pH 11.3) buffer. Alternatively, CRP monomer-bound cells were extracted using cold triton for 15 min (1% triton X-100, 4ºC). The residue binding of CRP monomer was assessed as described in the Materials and Methods section. $*p < 0.05$ compared to the control.

We further investigated whether the CRP monomer can influence foam cell formation. U937 monocytes were first transformed into macrophages with PMA. A similar binding pattern was observed for the CRP monomer binding to the differentiated macrophages exhibiting upregulated CD32 and negative CD16 (not shown). As shown in Fig. 4A, incubating n-LDL with U937 macrophages for 16 h did not result in foam cell formation. No apparent difference was observed when n-LDL was added together with the CRP monomer. By contrast, adding ox-LDL lead to dramatic foam cell formation as evidenced by the massive accumulation of lipid droplets in the cytoplasm, showing punctates and exceptionally bright Nile red staining [20]. The presence of the CRP monomer significantly reduced ox-LDL-induced intracellular lipid accumulation (~65% decrease in fluorescence intensities). In addition, the CRP monomer was also able to inhibit ox-LDL-induced lipid droplet accumulation in PBMC-

derived macrophages in a dose-dependent manner (Fig. 4B). These findings suggest that the CRP monomer was able to prevent foam cell formation.

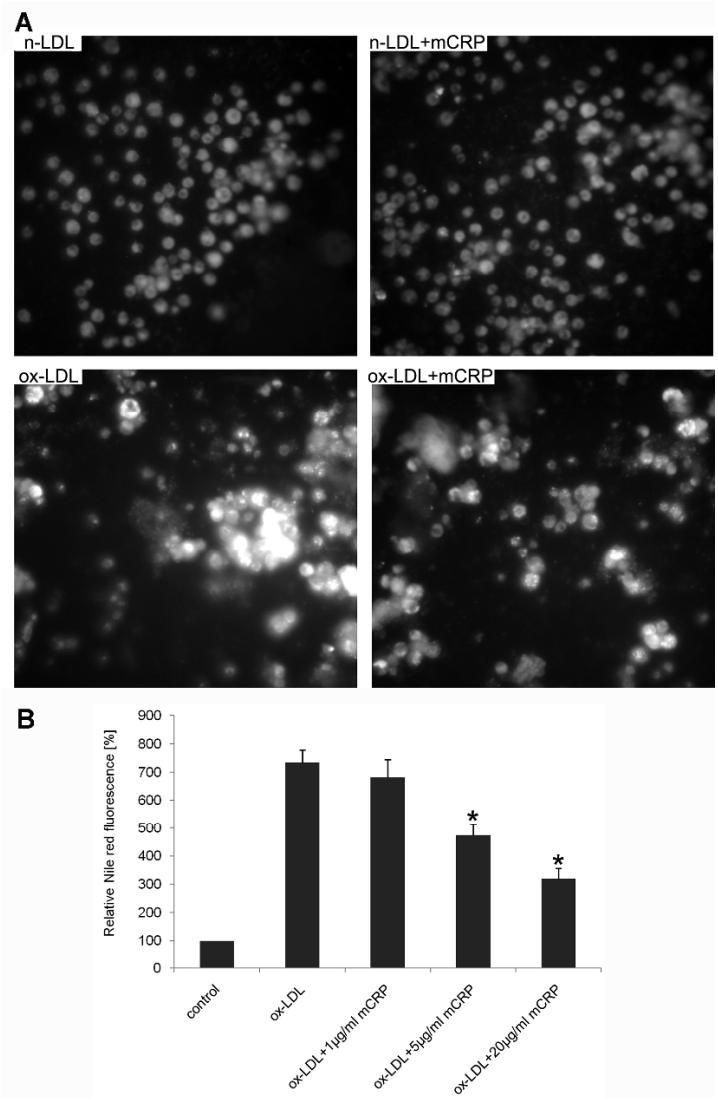

Fig. 4. The inhibition of ox-LDL-induced foam cell formation by the CRP monomer. A – U937 macrophages were incubated with 20 µg/ml native LDL (n-LDL), 20 µg/ml LDL + 20 µg/ml CRP monomer (mCRP), 20 µg/ml ox-LDL or 20 µg/ml ox-LDL + 20 µg/ml CRP monomer for 16 h. The levels of intracellular lipid droplet accumulation and foam cell formation were determined by Nile red staining. One representative result is shown. B – PBMC-derived macrophages were incubated with or without 20 µg/ml ox-LDL in the presence of CRP monomer at the indicated concentrations for 16 h. The fluorescence of Nile red staining of each sample was then quantified. *$p < 0.05$ compared to the control.

DISCUSSION

Although the CRP monomer evokes cellular responses from neutrophils through CD16, the existence of additional receptors or pathways on neutrophils [10, 11] and other cell types [7, 8] has been proposed. In this study, using a CD16-negative U937 cell line, we demonstrated a specific cellular binding of the CRP monomer in a FcγRs-independent manner, consistent with the previous findings using endothelial cells [25]. By contrast, the association of the CRP monomer with U937 monocytes seems to be mediated by incorporation into the plasma membrane rather than by binding to a cell surface ligand. This conclusion is supported by three lines of evidence:

1. enzymatic digestion of the surface protein by trypsin was less efficient at suppressing CRP monomer binding, suggesting a protease-insensitive mechanism;
2. non-proteinous glycosaminoglycan ligands failed to inhibit CRP monomer binding;
3. cell-bound CRP monomer could not be released by high salt, acid or alkaline extraction, again excluding the involvement of ligand-receptor interactions.

It is still possible that the CRP monomer may bind to a trypsin-insensitive transmembrane receptor with exceptionally strong affinity and thus be resistant to harsh acidic dissociation. Nevertheless, our findings highlight that the CRP monomer may directly interact with monocytes independently of CD16.

The uptake of modified LDL by monocyte-derived macrophages in plaque is the major route for foam cell formation, a critical event in atherogenesis [12]. Interestingly, we found that ox-LDL-induced accumulation of intracellular lipid droplets could be effectively inhibited by the CRP monomer. As the membrane binding of the CRP pentamer would lead to rapid conversion of the CRP monomer [6, 26], our data can be well reconciled with a recent report that the CRP pentamer can block foam cell formation induced by enzymatically modified LDL [27]. Together with the possible beneficial effects of the CRP monomer on complement regulation in plaques [28], our results suggest a protective role of the CRP monomer in atherogenesis, in agreement with the finding that the CRP monomer inhibits early progression of atherosclerosis in an $ApoE^{-/-}$ mice model [29]. Whereas these may be seemingly incompatible with the various pro-inflammatory actions of the CRP monomer on endothelial cells [7], platelets [8] and neutrophils [10], it would not be surprising for one protein to play different or even opposite roles at different stages of a disease. The balance of pro- and anti-atherosclerotic actions of the CRP monomer may be fine-tuned by the local microenvironment associated with the disease. Thus, extensive investigations will be required for a full understanding of the context-dependent contributions of the CRP monomer.

The CRP monomer was reported to form a complex with ox-LDL, and to inhibit its uptake by monocytes [30]. However, it is not likely that the < 30%

suppression in ox-LDL uptake [30] could account for the profound inhibition of the intracellular lipid droplet accumulation demonstrated herein. Rather, because the CRP monomer can incorporate into both the lipid shell of LDL particles [30] and the plasma membrane of macrophages, as shown in this study, these interactions may retard the cellular adsorption of lipids from LDL either at the cell surface or in the endocytic vesicles. Alternatively, the CRP monomer may directly change the profile of lipid metabolism in the monocytes. These interesting possibilities certainly deserve further investigation.

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