Loss of Pentameric Symmetry of C–Reactive Protein is Associated with Delayed Apoptosis of Human Neutrophils

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Running title: Monomeric C-reactive protein delays neutrophil apoptosis
Summary

Human neutrophil granulocytes die rapidly and their survival is contingent upon rescue from programmed cell death by signals from the environment. Here we report that a novel signal for delaying neutrophil apoptosis is the classic acute-phase reactant, C-reactive protein (CRP). However, this anti-apoptotic activity is expressed only when the cyclic pentameric structure of CRP is lost, resulting in formation of modified or monomeric CRP (mCRP), which may be formed in inflamed tissues. By contrast, native pentameric CRP and CRP peptides 77-82, 174-185 and 201-206 failed to affect neutrophil apoptosis. The apoptosis delaying action of mCRP was markedly attenuated by an antibody against the low affinity IgG immune complex receptor (CD16), but not by an anti-CD32 antibody. mCRP evoked a transient concurrent activation of the extracellular signal-regulated kinase (ERK) and phosphatidylinositol 3-kinase/Akt signaling pathways, leading to inhibition of caspase-3, and consequently to delaying apoptosis.

Consistently, pharmacological inhibition of either ERK or Akt reversed the anti-apoptotic action of mCRP; however, they did not produce additive inhibition. Thus, mCRP, but not pentameric CRP or peptides derived from CRP, promotes neutrophil survival and may therefore contribute to amplification of the inflammatory response.
Migration of neutrophil granulocytes into tissues during inflammation is intimately linked to their activation for functional activity as well as cell survival. Mature human neutrophils have the shortest life span among leukocytes and die rapidly via apoptosis in vitro and, apparently, in vivo (1-4). Neutrophils undergoing apoptosis lose CD16 (FCγRIII) expression (5,6) and show a reduced ability to respond to chemoattractants (3,4). This constitutively expressed program may serve to render neutrophils functionally ineffective before their removal by scavenger macrophages (3). However, the life span of mature neutrophils can be extended significantly within the inflammatory microenvironment by bacterial products (2,4), pro-inflammatory cytokines, including IL-2, IF-γ and GM-CSF (4), and glucocorticoids (7). The regulation of neutrophil apoptosis during the acute-phase of inflammation is less well defined, yet it is critical to the optimal expression and resolution of inflammation.

C-reactive protein (CRP), a prototypical acute-phase reactant, is a member of the pentraxin family of highly conserved cyclic pentameric proteins (8,9). Despite extensive studies spanning several decades, the exact role and mechanism of action of CRP as a modulator of inflammation has not been well defined, as both pro- and anti-inflammatory actions have been reported (8-15). These apparently contradictory results might be explained by formation of distinct species of CRP during inflammation. In general, the effects of native, pentameric CRP on neutrophils are largely inhibitory. CRP binds primarily to the low-affinity IgG FcγRIIa (CD32) and to some extent to the high-affinity IgG FcγRI (CD64) (16-18). Neutrophils exposed to native CRP show depressed functional activities, including degranulation (19), generation of superoxide by the inducible respiratory burst (19), adherence to endothelial cells (15), and migration into tissues (13). Conformationally altered and/or proteolytic forms of CRP express
several epitopes that are not present on native CRP (20), and display properties distinct from those of native CRP (21). Native, pentameric CRP can be dissociated into free subunits \textit{in vitro} (21). These subunits expressing several neoepitopes are referred to as modified or monomeric CRP (mCRP). mCRP antigens were detected in inflamed rabbit tissues (22) as well as in the wall of human normal blood vessels (23). Unlike native CRP, mCRP binds to the low-affinity IgG immune complex FcγRIIIb (G. Schneider \textit{et al}., personal communication) and enhances neutrophil adhesiveness to endothelial cells via up-regulation of the expression of CD11b/C18 on the neutrophil surface (24). Exposure of CRP to membrane-bound serine proteases leads to cleavage of biologically active peptides, which mimic the actions of native CRP on neutrophil chemotaxis (25) and adhesion to endothelial cells (15). In this study, we investigated whether CRP, mCRP or peptides derived from CRP could prolong neutrophil life span via inhibition of apoptosis.

\textbf{MATERIALS AND METHODS}

\textit{C-reactive protein and related proteins/peptides} - High purity (>99%) human native CRP (Calbiochem) was stored in buffers containing CaCl$_2$ to prevent the spontaneous formation of monomeric CRP from the native CRP pentamer. Monomeric or modified CRP (mCRP) was prepared from native CRP by urea chelation (21). By electron microscopy analysis, mCRP molecules in the absence of added salt associates into a diffuse matrix very distinct from the annular pentameric disk that is defined for native CRP (27). The secondary structure of native CRP has been estimated using X-ray crystallography (28) and Fourier transform infrared spectroscopy (29) as 50\% $\beta$-sheet, 12\% helix, 24\% $\beta$-turn and 14\% unordered structure. A preliminary evaluation of mCRP secondary structure using Circular dichroism and a self-
consistent method (Selcon) for estimating structures (30), revealed mCRP to have 50-51% helix, 2-12% β-sheet, 20-23% β-turn and 12-20% random structure. These data indicate that a significant secondary structural change occurs when mCRP is formed from CRP, changing from predominantly β-sheet structure to an α-helical structure. A recombinant form of mCRP (rmCRP) with both cysteine residues mutated to alanine residues (i.e. C₃₆ → A; C₉₇ → A) and with an added N-terminal formyl-methionine residue was expressed in *E. coli* and was isolated from inclusion bodies to >95% purity (26). To enhance solubility, rmCRP was acylated with maleic anhydride or citraconic anhydride. Cysteine-mutated rmCRP was directly comparable to mCRP produced from the native CRP pentamer by urea chelation in the following ways: (a) SDS-PAGE analysis showed both rmCRP and mCRP proteins displayed one predominant protein with an apparent molecular weight of 22,976, identical to that of a native CRP subunit; (b) amino acid composition analysis (Analytical Biotechnology Services, Boston, MA) showed that rmCRP contained the same number of amino acids residues per mole protein as mCRP with the exception of 11 alanine residues per mole of rmCRP compared to 9 per mole of mCRP, no cysteine residues in rmCRP versus 2 cysteine per mole of mCRP and 3 methionine residues per mole of rmCRP compared to 2 in mCRP, corroborating the changes engineered in rmCRP; (c) the N-terminal sequence analysis of rmCRP (pulsed liquid sequencer with an in-line phenyl-thiohydantoin amino acid analyzer, Analytical Biotechnology Services, Boston, MA) determined a sequence, MQTDMSRKAFVFPKE, that exactly corresponds to the sequence established for the CRP subunit (31,32); (d) monoclonal antibodies directed to the C-terminal octapeptide (residues 199-206) of the CRP subunit (clone 3H12 or 9C9) (20) react with the same specificity and affinity to mCRP, rmCRP (33), and acylated forms of rmCRP. Furthermore, preliminary experiments showed that, on a molar basis, mCRP and rmCRP and acylated forms of rmCRP produced similar delays
in neutrophil apoptosis assessed by annexin-V and acridine orange staining (see below).

Therefore, because of the enhanced solubility and the similarity in biochemical characteristics, most results described in this report were obtained with maleic anhydride-acylated rmCRP. CRP peptides 77-82, 174-185 and 201-206 (purity >98%) were obtained from Sigma Chemical Co. The endotoxin levels of CRP, mCRP, rmCRP and CRP peptide solutions were below the detection limit (0.125 EU/ml, corresponding to ~0.01 ng/ml Ec5) of the Limulus amebocyte lysate assay (E-Toxate, Sigma).

Isolation and culture of neutrophils - Neutrophils were isolated from venous blood of healthy volunteers (male and female, 22-48 years), who had not taken any drugs for at least 14 days before the experiments (15). The protocol was approved by the Clinical Research Committee of the Maisonneuve-Rosemont Hospital. Neutrophils (5x10^6 cells/ml, purity >97%, viability >98%) were resuspended in Hanks’ balanced salt solution (HBSS) supplemented with 10% autologous plasma, and incubated with native or modified CRP or peptides derived from CRP in 0.5 ml conical polypropylene tubes on a rotator (Adams Nutator) at 37°C in 5% CO2. In additional experiments, neutrophils were preincubated with 50 µM PD98059, 2 µM wortmannin, 1 µM SB 203580, anti-CD16 Ab 3G8, anti-CD62 Ab FLI8.26 or the irrelevant Ab MOPC-21 (each at 2.5 µg/ml, PharMingen) for 20 min before addition of mCRP. At the indicated times, cells were washed once in HBSS before use in the assays described below.

Assessment of apoptotic morphology - Percentage of apoptotic cells was calculated by examining acridine orange-stained neutrophils (at least 300 cells/sample) for morphologic features characteristic for apoptosis (diminution of cell volume, fragmented or bright, homogeneously
stained nuclei) on a Leitz fluorescence microscope (34). Standard cytospin preparations were stained with Wright-Giemsa dye for photographic demonstration of apoptotic morphologic features.

Quantification of apoptosis - Neutrophil apoptosis was quantitated as the percent of cells with hypodiploid DNA (35) and positive annexin-V staining (36). Neutrophils (~10⁶) were suspended in 0.5 ml hypotonic fluorochrome solution (50 µg/ml propidium iodide in 0.1% sodium citrate plus 0.1% Triton X-100) immediately before analysis. Propidium iodide fluorescence of individual nuclei was measured using a FACScan flow cytometer (Becton Dickinson). 10,000 events were acquired per sample. For specific annexin-V binding, neutrophils (10⁶) were incubated in 100 µl binding buffer (10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl₂, pH 7.4) containing a saturating concentration of phycoerythrin-labeled annexin-V for 15 min at 20°C and then washed with phosphate buffered saline before analysis by flow cytometry. Non-specific binding was determined by using calcium-free binding buffer (10 mM HEPES, 140 mM NaCl, 10 mM EDTA, pH 7.4).

DNA fragmentation - DNA cleavage was shown by quantification of fractional solubilized (low molecular weight) DNA by diphenylamine assay (37) and gel electrophoresis (34). For the diphenylamine assay, data are reported as the percentage of soluble, low molecular weight DNA. For DNA electrophoresis, DNA was extracted from neutrophils, precipitated and resuspended in Tris-EDTA buffer containing 25 µg/ml RNAse A (Roche), incubated for 5 min at 65°C, and then subjected to electrophoresis in 0.8% agarose at 80 V for 70 min (34). After staining with ethidium bromide, DNA was visualized by UV examination for image analysis.
Caspase-3 activity assay - Cell lysates, prepared from $10^7$ neutrophils, were incubated for 60 min at 37°C in 200 μl assay buffer containing 28 μM N-acetyl-Asp-Glu-Val-Asp-AMC (BD Biosciences). Release of AMC from Ac-DEVD-AMC was measured using a CytoFluor microplate reader (PE Biosystems) with excitation and emission wavelengths of 340 nm and 460 nm, respectively. Cleavage of pro-caspase-3 was assessed by western blotting using a polyclonal anti-caspase-3 antibody (PharMingen) that recognizes both the 32 kDa unprocessed pro-caspase-3 and the 17 kDa subunit of the active caspase-3.

Western blot analysis - Protein extracts were prepared by lysing 2x10^6 neutrophils in 100 μl lysis buffer, and western blot analysis of phosphorylated ERK 1/2, p38 MAPK and Akt was performed using the Phospho Plus ERK 1/2 and p38 MAPK, and Akt Ab kits (New England Biolabs) as described (38).

Statistical analysis - Results are expressed as means ± SEM. Statistical comparisons were made by ANOVA using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test to identify differences between various treatments. P values <0.05 were considered significant for all tests.

RESULTS

Modified CRP, but not native CRP or peptides derived from CRP, delays development of apoptotic morphology in neutrophils - Control (untreated) neutrophils developed prominent morphologic features of apoptosis, including loss of membrane asymmetry (assessed by annexin-V binding), diminution in cell volume, chromatin condensation and internucleosomal cleavage of
DNA, resulting in hypodiploid nuclei, within 24 hours of culture. Modified CRP inhibited the development of apoptotic morphology at each of the time points studied (Figs. 1 and 2). The concentration required to inhibit neutrophil apoptosis by 50% (EC\textsubscript{50}) was 6-8 µg/ml (0.26-0.35µM) (Figs 1b, 2c and 2e). The maximum inhibition that can be achieved with mCRP (50 µg/ml) was similar to that seen with lipopolysaccharide (LPS, 1 µg/ml) (Fig. 2a). Although mCRP-treated cells generally retained a non-apoptotic morphologic appearance after 24 to 48 hours in culture, nuclear condensation and decreased cell volume were evident in approximately 70% of neutrophils by 72 hours. By contrast, neither native CRP nor CRP peptide 201-206 affected significantly annexin-V binding (Fig. 1b) and the percentage of cells with apoptotic morphology (data not shown). Likewise, CRP peptides 77-84 and 174-185 were also ineffective (data not shown). Thus, among the CRP proteins/peptides tested, only mCRP appears to delay apoptosis in neutrophils. Furthermore, the apoptosis delaying action of mCRP can also be observed in an environment containing an excess of native CRP. For instance, 5 µg/ml mCRP reduced the percentage of annexin V-positive neutrophils from 49.4±7.9% to 19.9±4.0% or 20.8±3.5% in the absence or presence of 50 µg/ml native CRP, respectively after 24 h culture (n=3).

\textit{Modified CRP attenuates DNA fragmentation} - Because DNA fragmentation is considered to be a hallmark of apoptosis, we performed a quantitative assay of DNA fragmentation. Neutrophils cultured for 24 hours showed a dramatic increase in the amount of low molecular weight DNA from a control value of 2% (in freshly isolated neutrophils) to 32% (P<0.01). Neutrophils cultured with mCRP showed marked decreases in the proportion of low molecular weight (soluble) DNA (Fig. 2f). The effect of mCRP was concentration-dependent with an EC\textsubscript{50} value of
10 μg/ml. Electrophoretic analysis confirmed the ability of mCRP to inhibit DNA fragmentation in neutrophils (Fig. 2g). The effect of 20 and 100 μg/ml mCRP was comparable to those of 1 μg/ml LPS (Fig. 2g).

**mCRP delays neutrophil apoptosis through binding to FcγRIII (CD16)** - We used function-blocking antibodies (Abs) to CD16 and CD32 (FcγRIIa) as competitors to determine the IgG receptor subtype responsible for the apoptosis delaying action of mCRP. mCRP-induced decreases in annexin-V binding and percentage of neutrophils with apoptotic morphology were markedly attenuated in the presence of anti-CD16 Ab (Fig. 3). Neither the anti-CD32 Ab nor the irrelevant Ab MOPC-21 affected the mCRP inhibition (Fig. 3). None of the Abs by themselves affected neutrophil apoptosis (data not shown).

**mCRP activates the MAPKK/ERK and PI 3-kinase/Akt signaling pathways** - To assess the intracellular signaling pathways that mediate the apoptosis delaying action of mCRP, we studied the activation of various MAP kinases known to regulate cell survival. Neutrophils incubated with mCRP induced a transient, time and concentration-dependent increase in phosphorylation of ERK 1/2 and Akt relative to unstimulated controls (Fig. 4a and b). Phosphorylation of both ERK and Akt was rapid in onset, reaching a peak at around 2 min. The relative degree of ERK and Akt phosphorylation induced by formyl-Met-Leu-Phe (100 nM) is shown for comparison (Fig. 4b). **In vitro** culture of neutrophils was associated with a sustained activation of p38 MAPK (Fig. 4c). Enhanced p38 MAPK phosphorylation was detected as early as at 2 hours of culture, and was not blocked by either mCRP or LPS (Fig. 4c).
To assess the role of Akt, ERK and p38 MAP in mCRP-affected neutrophil apoptosis, we used the selective MAPK/ERK kinase inhibitor PD98059, the PI 3-kinase (the upstream regulator of Akt activation) inhibitor wortmannin, and the p38 MAPK inhibitor SB 203580. Neither PD98059, nor wortmannin alone affected development of neutrophil apoptosis, whereas SB 203580 significantly inhibited the percentage of annexin-V positive cells (P<0.01 compared to untreated control) and the percentage of neutrophils exhibiting hypodiploid nuclei (Fig. 4d). By contrast, both PD98059 and wortmannin effectively attenuated the inhibitory actions of mCRP, albeit complete reversal was never observed. The effects of PD98059 and wortmannin were not additive (Fig. 4d), indicating the mCRP effect is acting through a common downstream regulatory element. Combination of mCRP with SB 203580 did not result in greater degree of inhibition of neutrophil apoptotic features than those observed with mCRP or SB 203580 alone (Fig. 4d).

Increased caspase-3 activity during neutrophil apoptosis and its inhibition by mCRP - Caspase-3 activity was barely detectable in freshly isolated neutrophils, whereas in vitro culture of neutrophils for 24 h induced significant increases in caspase-3 formation and activity (Fig. 5). Cleavage of pro-caspase-3 was confirmed by Western blotting, which also demonstrated a pronounced increase in the 17 kDa active form of caspase-3 (Fig. 5a). Cleavage of pro-caspase-3 as well as caspase-3 activity were effectively reduced by mCRP in a concentration-dependent fashion (Fig. 5a and 5b, respectively). PD98059 and wortmannin attenuated the caspase-3 inhibitory action of mCRP (Fig. 5c). However, complete reversal was not achieved, not even with the combination of PD98059 and wortmannin.
DISCUSSION

Progression to apoptosis appears to be the normal default state for neutrophils, and cell survival is contingent upon rescue of cells from programmed cell death by signals from the environment. We describe in this report that a novel signal for delaying neutrophil apoptosis is the prototypical acute-phase reactant CRP. However, this action is “hidden” in native CRP, and is expressed only when the pentameric structure dissociates and undergoes a conformational rearrangement.

A growing body of evidence indicates that distinct species of CRP are formed during inflammation. Pentameric native CRP is synthesized and secreted mainly by hepatocytes into the blood (8,9). Pentameric CRP can be dissociated into free subunits through non-enzymatic chemical manipulations in vitro (21) and is apparently formed in vivo by a yet unidentified mechanism. Recent studies suggested that binding of native CRP to phosphorylcholine monolayers induces dissociation and re-arrangement of the subunits of native CRP, leading to exposure of an inter-subunit domain (39). Inter-subunit contact residues of native CRP include residues 115-123, 40-42 and 197-202 (28), the latter sequence of which is a predominant epitope of mCRP (20). The biochemical properties of the free subunits of CRP formed in vivo have not yet been described. Intriguingly, mCRP cross-reactive epitopes have been reported in vascular tissues (23), inflamed rabbit liver and muscle (22), monocytes (40) and islet cells (41). In addition, the genetic message for CRP subunit is expressed in peripheral blood mononuclear cells (40,42), alveolar macrophages (43), islet cells (41) and epithelial cells of the respiratory tract (44). Hence, the mCRP used in the present studies may be similar, if not identical to a naturally occurring isomeric form of CRP found in various tissues and cells throughout the body.
CRP and/or mCRP may be cleaved at one or more of three tuftsin-like (Thr-Lys-Pro-Arg) regions in CRP and mCRP, resulting in the release of biologically active peptides (45). Interestingly, mCRP is more susceptible to proteolysis than is the native CRP molecule (46).

In these studies we show that mCRP, but not native CRP or peptides derived from CRP, delays neutrophil apoptosis, even in the excess of native CRP. The mCRP action was demonstrable at low microgram per milliliter concentrations. Consistent with the commitment of neutrophils to apoptotic death, mCRP delayed, rather than blocked apoptosis, resulting in prolonged neutrophil survival in vitro. Thus, mCRP exerts an anti-apoptotic effect similar to those of G-CSF, glucocorticoids or LPS (2,4,7). Our results suggest that this action of mCRP is predominantly mediated through the low affinity immune complex binding IgG FcR, FCγRIIIb (CD16), for the function-blocking anti-CD16 Ab, but not anti-CD32 Ab, markedly, though never completely, reversed the anti-apoptotic action of mCRP. These observations are consistent with recent receptor binding studies, identifying FCγRIIIb as the primary binding site for mCRP on human neutrophils (G. Schneider et al., personal communication). However, we cannot exclude the possibility that the mCRP effect may be mediated through some yet undefined cell surface receptor(s). It is unlikely that mCRP might activate the low-affinity IgG FCγRIIa (CD32) or the high-affinity IgG FcγRI (CD64), because native CRP, which binds to these receptors (16-18), did not affect significantly development of neutrophil apoptosis. Intriguingly, neutrophils lose their surface FCγRIIIb during apoptosis (5,6), receptors which are capable of transmitting an anti-apoptotic signal as demonstrated in the present study. The receptors for peptides derived from CRP have not been identified to date, though the similarities in the biological actions of native CRP and peptides 77-82, 174-185 and 201-206 (refs.15 and 26, and the present study) would suggest involvement of the same receptors.
Several studies have reported the opposing effects of p38 MAPK and ERK or PI 3-kinase on apoptosis. In general, p38 MAPK appears to promote, while both ERK and PI 3-kinase appear to inhibit programmed cell death (47-50). Akt mediates the anti-apoptotic action of PI 3-kinase (51,52). Consistent with previous observations (48), our study also showed that spontaneous apoptosis in human neutrophils is associated with prolonged activation of p38 MAPK, and can be partially reversed by the specific p38 MAPK inhibitor SB203580. Activation of p38 MAPK then leads to cleavage of procaspase-3, yielding active caspase-3, one of the key effectors of apoptosis.

Incubation of neutrophils with mCRP led to rapid (within 1 to 2 min) and concentration-dependent activation of both ERK and Akt. In the present study, neither ERK nor PI 3-kinase inhibition could fully reverse mCRP-dependent inhibition of caspase-3 activation and delay the development of apoptotic morphology, suggesting an additional signaling mechanism at work. Furthermore, combination of PI 3-kinase and ERK inhibition did not completely reverse the effects of mCRP on apoptosis. In addition, neither PD98059 nor wortmannin inhibited phosphorylation of p38 MAPK in neutrophils undergoing spontaneous apoptosis. This would indicate that these pathways may converge downstream of p38 MAPK and are not working independently in delaying apoptosis. Signal transduction initiated by the glycosyl phosphatidylinositol-anchored FcγRIIIb leads to calcium mobilization and translocation of Src-related tyrosine kinases (53). Tyrosine kinase activation can lead to activation of ERK through the Ras/Raf-1/MEK pathway (24) or indirectly through activation of PI 3-kinase and Akt, as was reported for GM-CSF-activated signaling in neutrophils (49). Phosphorylated Akt has been shown to inactivate procaspase-9, a trigger of the activation of the caspase cascade (54). Our results indicate that inhibition of MEK/ERK signaling pathway clearly results in inhibition of
cleavage of pro-caspase-3 and caspase-3 activity. Therefore, mCRP could inhibit apoptosis by inactivating caspase-3 via activation of Akt and ERK. Homo and heterotypic cross-linking of FcγRIIa and FcγRIIIb was found to induce transient activation of Akt through PI 3-kinase in human neutrophils (55). However, native CRP, which binds to FcγRIIa but not to FcγRIIIb, did not affect significantly development of neutrophil apoptosis. This would suggest that Akt activation per se may not be sufficient to delay neutrophil apoptosis, rather induction of both the ERK and PI 3-kinase/Akt pathways is necessary to mediate neutrophil survival. Previously, we reported that native CRP does not activate ERK in neutrophils (24). Of interest, calcium pyrophosphate dihydrate crystals-induced repression of neutrophil apoptosis is also mediated through concurrent activation of ERK and PI 3-kinase/Akt (56). Activated Akt can phosphorylate BAD, a member of the Bcl-2 family (57). Phosphorylated BAD dissociates from Bcl-2, thereby enhancing the anti-apoptotic effects of the Bcl-2 family proteins (55). These results would suggest that mCRP induction of the ERK and PI 3-kinase pathways probably act in concert to repress caspase-3 activation via regulating expression of anti-apoptotic genes and/or by post-translational modification to inactivate the neutrophil intrinsic pro-apoptotic machinery. These specific mechanisms remain to be elucidated and are currently under investigation in our laboratory.

Our results may have relevance to neutrophil survival required for excessive leukocyte trafficking into inflamed tissues. While native CRP does not repress neutrophil apoptosis, it binds to the surface membranes of intact apoptotic cells and protects the cell from the assembly of the terminal complement membrane attack complex (58), thereby promoting non-inflammatory clearance of apoptotic cells. On the other hand, contact of mCRP with loosely attached neutrophils will not only lead to neutrophil activation and promotion of firm adhesion (24), but
also to repression of neutrophil apoptosis, thereby amplifying the acute inflammatory response.

We propose that endothelial injury may result in exposure of mCRP that is naturally expressed in the intima of blood vessels (23), and tissue injury or infection may lead to de novo formation of mCRP at the inflamed sites. Furthermore, considerable neutrophil-mediated degradation of mCRP may also occur at the same sites at later stages of inflammation, resulting in formation of CRP peptides 77-82, 174-185 and 201-206. Like native pentameric CRP, these peptides cannot retard development of neutrophil apoptosis, rather they might inhibit further recruitment of neutrophils (15,45), thereby contributing to demarcation of the inflammatory locus and resolution of inflammation.

In summary, our results show that structural rearrangement in the classical acute-phase protein CRP can prolong neutrophil survival in vitro. Indeed, loss of pentameric symmetry in CRP is associated with appearance of a novel apoptosis delaying bioactivity in mCRP. This action is mediated, in part, via activation of the low affinity IgG immune complex receptor (CD16) through stimulation of the PI 3-kinase/Akt and MEK/ERK signaling pathways, leading to inhibition of caspase-3 (Fig. 6). Thus, mCRP, but not native CRP or peptides derived from CRP, promotes neutrophil survival and may therefore contribute to amplification of the inflammatory response.
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Footnotes

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1Abbreviations used in this paper: CRP, C-reactive protein; mCRP, modified or monomeric CRP; Ab, antibody; LPS, lipopolysaccharide; ERK 1/2, extracellular-signal regulated kinase 1/2; MAPK, mitogen-activated protein kinase; PI 3-kinase, phosphatidylinositol 3-kinase
Figure legends

FIG. 1. mCRP, but not native CRP or CRP peptide 201-206, delays neutrophil surface expression of phosphatidylserine (PS) (assayed by annexin-V staining). (a) Time course of PS expression in neutrophils maintained in vitro in the absence or presence of native CRP or mCRP (50 µg/ml). (b) Concentration-dependent inhibition of PS expression by mCRP. Aliquots of neutrophil suspensions were cultured with one of the proteins/peptide for 24 h. Values are the mean ± SEM of 4-7 separate experiments using neutrophils prepared from different donors. *P<0.05; **P<0.01 (compared with untreated control).

FIG. 2. mCRP delays neutrophil apoptosis. (a) Morphologic features of neutrophils maintained in suspension culture. Cytospin preparations of neutrophils were prepared and stained immediately after isolation of neutrophils (0h) and after incubation in vitro for 24 hours in the absence (untreated) or presence of mCRP (50 µg/ml) or LPS (1 µg/ml). (b-e) Kinetic analysis and concentration dependence of the effects of mCRP on development of apoptotic morphology. Aliquots of neutrophil suspensions were prepared and scored for apoptotic morphology (b and c) or were processed for nuclear DNA content analysis (d and e). Results are the mean ± SEM for 5-7 experiments using neutrophils from different donors. *P<0.05; **P<0.01 (compared with untreated control). (f) DNA fragmentation is reported as the ratio of DNA cleavage products to total DNA expressed as percentage. Results represent the mean ± SEM of four separate experiments performed with neutrophils isolated from different blood donors. * P<0.05, ** P<0.01 (compared with untreated). (g) mCRP attenuates chromatin cleavage in neutrophils maintained in culture for 24 h. The effect of LPS (1 µg/ml) is shown for comparison.
The left lane represents DNA kilobase marker standards, and values of selected standards are shown on the left margin. The results are representative for four independent experiments.

FIG. 3. **Anti-CD16 Ab reverses the apoptosis-delaying action of mCRP.** Isolated neutrophils were maintained *in vitro* for 24 h in the absence and presence of mCRP (50 µg/ml) plus an anti-CD16 Ab, anti-CD32 Ab or the irrelevant Ab MOPC-21. Aliquots of neutrophil suspensions were stained with annexin-V or were processed for nuclear DNA content analysis. Results are the mean ± SEM for five experiments using neutrophils from different donors. * P<0.05; **P<0.01 (compared with control untreated (open columns).

FIG. 4. **mCRP inhibits neutrophil apoptosis by activation of the ERK and Akt signaling pathways.** Isolated neutrophils were challenged with mCRP (50 µg/ml) or LPS (1 µg/ml) for the indicated times (a and c) or with various concentrations of mCRP or formyl-Met-Leu-Phe (10^{-7} M) for 2 min (b). (a-c) Western blot analysis of phosphorylated kinases. Proteins were isolated and probed with phospho-specific Abs. β-actin and total p38 MAPK served as control for equal protein loading. The results are representative of four independent experiments. (d) Inhibition of MAPK/ERK kinase and PI 3-kinase attenuates the apoptosis delaying action of mCRP. After 24 h incubation, aliquots of neutrophil suspensions were stained with annexin-V or were processed for nuclear DNA content analysis. Results are the mean ± SEM of six experiments using neutrophils from different donors. * P<0.05 (compared with control untreated (open columns); # P<0.05; ## P<0.01 (compared with mCRP alone).
FIG. 5. **mCRP decreases caspase-3 activity in human neutrophils.** Whole cell extracts were prepared from freshly isolated neutrophils (T₀) or from neutrophils incubated with mCRP for 24 h. (a) Immunoblotting for procaspase-3 processing. The antibody recognizes both pro-caspase 3 (32 kDa) and caspase-3 (17 kDa). The results are representative of four experiments using neutrophils from different donors. (b) Concentration-dependent inhibition of caspase-3 activity. Caspase-3 activity was determined using AC-DEVD-AMC as a substrate. No fluorescence was detected in the presence of Ac-DEVD-CHO, an inhibitor of caspase-3 activity. (c) Reversal of the effect of mCRP on caspase-3 activity by PD98059 and wortmannin. Values are the mean ± SEM of four independent experiments. * P<0.05; **P<0.01 (compared with mCRP alone).

FIG. 6. **Proposed mechanism for the effect of mCRP on neutrophil apoptosis.** Neutrophil survival may be controlled by the opposing actions of the ERK, Akt and p38 MAPK pathways. Constitutive neutrophil apoptosis is associated with activation of p38 MAPK and consequently caspase-3. In the presence of mCRP, a survival signaling pathway (through ERK and Akt) is activated, leading to inhibition of caspase-3 formation/activity and delay of apoptosis.
% Cells with apoptotic nuclei

% Annexin-V positive cells

mCRP

anti-CD16

anti-CD32

MOPC-21

P < 0.01
Pentameric native CRP

Peptides 77-82, 174-185, 201-206

mCRP

anti-CD16 Ab

Aging

Apoptotic signal

SB 203580

p38 MAPK

Erk

Akt

Caspase 3

Apoptosis

PD 98059

MAPKK

PI3-kinase

wortmannin

FcγRIIib
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