Opposing Effects of C-Reactive Protein Isoforms on Shear-Induced Neutrophil-Platelet Adhesion and Neutrophil Aggregation in Whole Blood

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Background—Plasma C-reactive protein (CRP) level is a powerful predictor of cardiovascular events. However, it is not known whether CRP could affect neutrophil-platelet adhesion and neutrophil aggregation, key events in acute coronary syndromes. Emerging in vitro evidence suggests that some bioactivities of CRP are expressed on loss of the pentameric symmetry, resulting in formation of modified or monomeric CRP (mCRP).

Methods and Results—We studied the impact of human native CRP and bioengineered mCRP that cannot rearrange into the pentameric structure on the kinetics of neutrophil-platelet adhesion and neutrophil aggregation in whole blood subjected to shear (≈100 s⁻¹) using real-time flow cytometry. Shear resulted in upregulation of platelet P-selectin expression, leading to platelet capture of neutrophils and subsequent neutrophil aggregation, which was dependent on P-selectin, L-selectin, and CD18. Native CRP at clinically relevant concentrations markedly attenuated these changes. The residual amount of neutrophil adhesion was blocked with anti-CD18 or anti-CD11b antibody. By contrast, mCRP concentration-dependently enhanced shear-induced platelet P-selectin expression and increased the rate and extent of formation of both neutrophil-platelet and neutrophil-neutrophil aggregates. Complete abrogation of platelet-neutrophil adhesion and neutrophil aggregation required both anti–P-selectin and anti-CD18 antibodies but not anti–L-selectin antibody. The CRP action was markedly inhibited by an anti-CD32 antibody, whereas the mCRP effects were significantly attenuated by an anti-CD16 antibody.

Conclusions—These results indicate that native CRP inhibits platelet activation and prevents platelet capture of neutrophils, whereas mCRP displays potent prothrombotic activities under low levels of shear. Thus, mCRP rather than native CRP may precipitate acute coronary syndromes. (Circulation. 2004;110:2713-2720.)

Key Words: neutrophils ■ platelets ■ cell adhesion molecules ■ inflammation ■ heart disease

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In the present study, we investigated the impact of native CRP and mCRP on the dynamics and molecular constituents that support neutrophil-platelet and neutrophil-neutrophil aggregates in human whole blood using real-time flow cytometry. Our results indicate that native CRP inhibits, whereas mCRP accelerates, hydrodynamic shear-induced platelet-neutrophil adhesion and subsequently neutrophil aggregation.

## Methods

### CRP Isoforms

High-purity (>99%) human native CRP (Calbiochem) was stored in Na3HPO4-free 20 mmol/L Tris, 150 mmol/L NaCl buffer (pH 7.5) containing 2 mmol/L CaCl2 to prevent spontaneous formation of mCRP from the native pentamer. A recombinant form of mCRP (r-mCRP; purity, >97%) that cannot rearrange into a pentameric structure was engineered and characterized as described.25 Native CRP was distinguished from mCRP by binding and antigenicity differences25 and by their secondary structure.25 The endotoxin level of all peptide solutions was below the detection limit (0.125 endotoxin units/mL, corresponding to 0.01 ng/mL lipopolysaccharide (LPS) of the Limulus assay (Sigma)).

### Detection of Neutrophil-Platelet Conjugates by Flow Cytometry

Venous blood (10 mL, anticoagulated with sodium heparin, 50 U/mL) was obtained from healthy, nonsmoking volunteers (male and female, 23 to 55 years old; leukocyte count, 3800 to 8800/μL) who had denied taking any medication for at least 2 weeks. The Clinical Research Committee approved the experimental protocols. Blood was kept at 20°C and used within 2 hours of collection. Two-hour storage of blood resulted in slight neutrophil activation, the order of measurements within each individual being randomized. Blood obtained from the same persons (at different times) was used to study both CRP and mCRP.

Formation of neutrophil aggregates and neutrophil-platelet conjugates was monitored in real time in accordance with the technique of Konstantopoulos et al28 with modifications. In brief, blood (100 μL) was diluted 1:6 with HEPES buffer. Platelets were labeled with an anti–CD61-FITC mAb followed by staining of leukocytes with LDS-751. A Control (unsheared) blood sample at 0 minutes showing single neutrophils with and without platelets. An LPS-751 fluorescence threshold (dashed line) was set to exclude unbound erythrocytes and single platelets from display. Sample was then subjected to hydrodynamic shear (100 s⁻¹) at 37°C for 14 minutes. Vertical line (FITC-fluorescence threshold) separates single neutrophils (left) from neutrophil aggregates (right). Horizontal line separates single neutrophils from neutrophil aggregates. B, Representative histogram of neutrophil aggregates after 14 minutes of shear. LPS-751 mean fluorescence values for neutrophil doublets, triplets, and quartets and larger aggregates are integral multiples of singlet value. RFU, relative fluorescence units.

Figure 1. Real-time flow cytometry analysis of neutrophil-platelet and neutrophil-neutrophil aggregates. Whole blood was diluted 1:6 with HEPES buffer. Platelets were labeled with an anti–CD61-FITC mAb followed by staining of leukocytes with LPS-751. A, Control (unsheared) blood sample at 0 minutes showing single neutrophils with and without platelets. An LPS-751 fluorescence threshold (dashed line) was set to exclude unbound erythrocytes and single platelets from display. Sample was then subjected to hydrodynamic shear (100 s⁻¹) at 37°C for 14 minutes. Vertical line (FITC-fluorescence threshold) separates single neutrophils (left) from neutrophil aggregates (right). Horizontal line separates single neutrophils from neutrophil aggregates. B, Representative histogram of neutrophil aggregation after 14 minutes of shear. LPS-751 mean fluorescence values for neutrophil doublets, triplets, and quartets and larger aggregates are integral multiples of singlet value. RFU, relative fluorescence units.
platelets adherent to their surface (95 ± 2%) were significantly (P < 0.01) or that were in aggregates of ≥2 cells (Figure 2, B and C). No changes were detected in the absence of shear (Figure 2, B and C).

Preincubation of blood samples for 5 minutes with native CRP produced decreases in neutrophil-platelet adhesion and neutrophil aggregation at all time points under shear without affecting the kinetics of adhesion (Figure 2, A–C). The CRP inhibition was concentration-dependent; statistically significant inhibition was detected even with 1 to 5 μg/mL, which peaked at 25 μg/mL (Figure 2, D and E). CRP effectively inhibited the adhesive events even when it was added on initiation of shear, although the degree of inhibition was somewhat lower than that detected after preincubation with CRP (data not shown). Heat inactivation of CRP (30 minutes at 100°C) resulted in a complete loss of its activity (data not shown). Because preincubation of unheated blood with mCRP for 10 minutes already produced detectable increases in cell adherence, mCRP was added to blood on application of shear. mCRP (25 μg/mL) enhanced the rate and extent of formation of neutrophil-platelet and neutrophil-neutrophil conjugates (Figure 2, B and C). Platelet adhesion to neutrophils reached a plateau at ≈5 minutes (Figure 2B). At 14 minutes of shear mixing, the number of neutrophils that had platelets adherent to their surface (95 ± 1%) or that were in aggregates of ≥2 cells (78 ± 2%) were significantly (P < 0.01) higher than those subjected to shear only. The mCRP actions were concentration-dependent; statistically significant potentiation was detected with 1 μg/mL, which peaked at 25 μg/mL (Figure 2, D and E). Addition of LPS (Escherichia coli serotype O111:B4, 0.02 ng/mL; a concentration ≥2-fold higher than the maximum level of LPS detected in our peptide solutions) did not affect shear-induced platelet-adhesion events of shear. mCRP (25 μg/mL) was added to blood on application of shear, which already produced detectable increases in cell adherence, mCRP was added to blood on application of shear. mCRP (25 μg/mL) enhanced the rate and extent of formation of neutrophil-platelet and neutrophil-neutrophil conjugates (Figure 2, B and C). Platelet adhesion to neutrophils reached a plateau at ≈5 minutes (Figure 2B). At 14 minutes of shear mixing, the number of neutrophils that had
neutrophil or neutrophil-neutrophil adherence. For instance, at 14 minutes of shear, 89±2% and 90±3% of neutrophils were adhered to platelets, and 48±3% and 49±5% of neutrophils were in aggregates in the absence and presence of LPS, respectively, n=5, P>0.1.

Opposing Effects of Native CRP and mCRP on Hemodynamic Shear-Induced Expression of Platelet P-Selectin
Both platelet P-selectin expression and the number of platelet bound to neutrophils increased with time of shear, as evidenced by the 1.7-fold increase in P-selectin fluorescence and the 4-fold increase in platelet CD61-FITC fluorescence of single neutrophils that were bound to platelets, respectively, after 14 minutes of shear (Figure 3). Preincubation of blood samples with native CRP concentration-dependently attenuated these events, indicating inhibition of platelet activation and adherence (Figure 3). Statistically significant inhibition was detected with 5 μg/mL CRP. In the presence of 25 μg/mL CRP, P-selectin expression after 14 minutes of shear did not differ from that of unshaved (time 0) platelets (Figure 3B). By contrast, mCRP concentration-dependently increased the rate and extent of CD62P expression and augmented CD61-FITC fluorescence intensity associated with neutrophil-bound platelets (Figure 3, B and D). A statistically significant increase was detected with 5 μg/mL mCRP, which peaked at 25 μg/mL.

Dependence of Homotypic and Heterotypic Neutrophil Adhesion on Selectins and β2-Integrins
To assess the contribution of multiple adhesion molecules to neutrophil-platelet and neutrophil-neutrophil adhesion in sheared suspensions, we used function-blocking mAbs. Anti-P-selectin mAb decreased the rate of adhesive interactions, resulting in ~30% inhibition of neutrophil-platelet adhesion and ~55% inhibition of neutrophil-neutrophil conjugates after 14 minutes of shear (Figure 4). Blocking of either CD18 or L-selectin alone did not significantly decrease either neutrophil-platelet or neutrophil-neutrophil adhesion (Figure 4). However, a combination of anti-P-selectin and anti-L-selectin mAbs resulted in 64% inhibition of neutrophil-platelet adhesion after 14 minutes of shear (Figure 4). Simultaneous blockade of P-selectin and CD18 almost completely inhibited neutrophil-platelet adhesion and reduced neutrophil aggregation by ~73% (Figure 4).

In CRP-treated blood samples, P-selectin blockade produced only slight decreases, whereas simultaneous P-selectin and CD18 blockade resulted in significant decreases in cell-cell interactions compared with those of native CRP alone (Figure 4). L-selectin blockade was without effect. A combination of native CRP with anti-P-selectin and anti-CD18 mAbs yielded almost complete blockade of neutrophil-platelet adhesion and inhibited neutrophil homotypic adhesion by ~75% after 14 minutes of shear (Figure 4).

The mCRP-accelerated neutrophil-platelet adhesion and neutrophil aggregation were significantly reduced at all time points under shear in the presence of anti-P-selectin mAb, whereas neither anti-L-selectin nor anti-CD-18 mAb alone produced significant inhibition (Figure 4). A combination of anti-P-selectin mAb and anti-L-selectin mAb did not result in significantly greater inhibition than that observed with anti-P-selectin mAb alone (Figure 4). A combination of anti-P-selectin and anti-CD18 mAbs inhibited neutrophil-platelet adhesion by ~94% and neutrophil aggregation by ~81% after 14 minutes of shear (Figure 4).

Blocking Mac-1 with anti-CD11b mAb alone did not yield significant inhibition (Figure 5). However, a combination of anti-P-selectin and anti-Mac-1 mAbs was as effective as anti-P-selectin plus anti-CD18 mAbs in blocking platelet-neutrophil and neutrophil-neutrophil adhesion (Figure 5).

Involvement of CD16 and CD32 in Native CRP and mCRP Signaling
We used function-blocking mAbs to CD16 (FcγRIII) and CD32 (FcγRIIa) as competitors to determine the IgG receptor
subtype mediating the actions of mCRP and CRP, respectively.32,33 None of these antibodies by themselves significantly affected the parameters studied (Figure 6). The anti-CD32 mAb effectively, although never completely, prevented the inhibitory action of CRP on shear-induced neutrophil-platelet adhesion, neutrophil aggregation, CD61-FITC fluorescence of single neutrophils, and platelet P-selectin expression, whereas the anti-CD16 mAb was without detectable effects (Figure 6). Conversely, the anti-CD16 mAb, but not the anti-CD32 mAb, significantly, although never completely, attenuated the effects of mCRP (Figure 6). The irrelevant antibody MOPC-21 had no detectable effects on the responses to either CRP or mCRP (data not shown).

**Discussion**

Numerous epidemiological studies have shown that plasma CRP level is a powerful predictor of future cardiovascular diseases.1–3 However, the pathophysiological importance of CRP is far from being fully understood. The present study provides evidence for a dual role of CRP isoforms in the regulation of shear-induced platelet-neutrophil adhesion, neutrophil aggregation, CD61-FITC fluorescence of single neutrophils, and platelet P-selectin expression, whereas the anti-CD16 mAb was without detectable effects (Figure 6). Conversely, the anti-CD16 mAb, but not the anti-CD32 mAb, significantly, although never completely, attenuated the effects of mCRP (Figure 6). The irrelevant antibody MOPC-21 had no detectable effects on the responses to either CRP or mCRP (data not shown).

We used a flow cytometry assay to monitor real-time transition of platelets from the “resting” to activated state and to study the dynamics of platelet adhesion to neutrophils and formation of neutrophil aggregates,28,30 as may occur in the circulating blood of patients with ACS. Apparently more neutrophils are adhered to platelets in diluted heparinized blood (as reported by previous28–31 studies and the present studies) than in blood collected into EDTA and formaldehyde.34 These discordant results might be attributed to differences in blood collection and handling rather than to a significant platelet activation, because we detected low levels of platelet P-selectin expression in unsheared samples. Shear mixing of diluted blood samples evokes negligible platelet aggregation,28 thereby eliminating the possibility of passive neutrophil entrapment in platelet aggregates. Low levels of hydrodynamic shear induce platelet activation, as evidenced by enhanced P-selectin expression. Likely mechanisms for platelet activation may involve hemolysis and release of ADP28,35; contact with foreign surfaces and altered O2 levels may function as additional signals. Activated platelets then bind to neutrophils, and this heterotypic cell interaction is a prerequisite for neutrophil aggregation.28 Our results show that native CRP concentration-dependently attenuated upregulation of P-selectin and consequently platelet adhesion to neutrophils and neutrophil aggregation. The inhibition was detectable at CRP levels of 1 to 5 g/mL, which are useful for predicting cardiovascular risk.2,3 Native CRP failed to affect the percentage of neutrophils that were bound to platelets, although it reduced neutrophil-associated platelet CD61-FITC fluorescence, indicating decreases in the number of platelets attached to single neutrophils. Flow cytometry did not allow determination of the number of platelets bound per neutrophil and assessment of whether platelets might have attached to neutrophils as single cells or small aggregates. Electron microscopy studies showed that most of the neutrophils were adherent to at least 2 platelets and that platelets either formed bridges between neutrophils or attached to a periphery away from the neutrophil-neutrophil contact region.28 These observations support the notion that critical
numbers of bound platelets (>2) may be necessary to induce neutrophil activation.28 The CRP inhibition of P-selectin expression is consistent with its inhibitory action on platelet reactivity in response to various agonists, including ADP.20–22 These findings raise the possibility that increased thrombus formation after arterial injury in human CRP-transgenic mice23 or left ventricular thrombus formation in patients with myocardial infarction could be attributed to distinct isoforms of CRP, perhaps directly derived from CRP, rather than to native CRP itself.

Native, pentameric CRP dissociates into free subunits after binding to plasma membranes or in a denaturing or oxidative environment, yielding mCRP. This is accompanied by a loss of predominantly $\beta$-sheet secondary structure, with an increase in $\alpha$-helix and expression of neoepitopes and distinct biological activities.24–27 Our study revealed that mCRP, unlike native CRP, induced P-selectin expression and enhanced the dynamics and extent of neutrophil-platelet adhesion and neutrophil aggregation during shear. Oxidized or heat-aggregated (ie, structurally modified) CRP has previously been reported to induce human platelet activation and aggregation in platelet-rich plasma in vitro.22,38 Of note, endotoxin levels in our CRP preparations were lower than the median plasma endotoxin value in a random population of men and women.39 However, neither LPS (at a concentration 2-fold higher than might be present in our protein preparations) nor heat-inactivated CRP evoked detectable effects, indicating that CRP or mCRP signaling was responsible for the observed effects.

Platelet-neutrophil adhesion is modeled as a multiple-step process.29,40 Platelet P-selectin binding to PSGL-1 on neutrophils mediates initial tethering, followed by their firm adhesion dependent on CD11b/CD18 (Mac-1) binding to GPIb and/or junctional adhesion molecule-3.42 In agreement with previous reports,28,40 we also found that P-selectin is crucial for platelet capture of neutrophils, and only combined P-selectin and CD18 or Mac-1 blockade resulted in almost complete abrogation of neutrophil-platelet adhesion under shear. L-selectin is also involved in this interaction, because a combination of anti-L-selectin and anti-P-selectin mAbs was more effective at blocking platelet adhesion than anti-P-selectin antibody alone. In the presence of native CRP, anti-P-selectin mAb did not produce a significant additive inhibition, indicating that prevention of P-selectin expression or interference with P-selectin binding by native CRP was critical for inhibition of subsequent cell-cell adhesion. In the

**Figure 5.** Dependence of platelet-neutrophil and neutrophil-neutrophil adhesion on Mac-1 (CD11b). Diluted blood was incubated for 10 minutes with a panel of blocking mAbs, as indicated. Samples were preincubated with native CRP (25 $\mu$g/mL) for 5 minutes. mCRP (25 $\mu$g/mL) was added on initiation of shear. Adhesion kinetics was monitored for neutrophil-platelet conjugates (A) and neutrophil aggregation (B). Values are mean ± SEM for 5 experiments using blood from different donors. *$P<0.05$, **$P<0.01$ vs shear (no antibodies). Comparisons were made separately for each group (ie, shear only or mCRP-treated samples).

**Figure 6.** Involvement of CD32 in CRP signaling and CD16 in mCRP signaling. Diluted blood was incubated for 10 minutes with function-blocking anti-CD16 or anti-CD32 mAb, as indicated. Samples were preincubated with native CRP (25 $\mu$g/mL) for 5 minutes. mCRP (25 $\mu$g/mL) was added on initiation of shear. Adhesion kinetics was monitored for neutrophil-platelet conjugates (A), neutrophil aggregation (B), mean platelet CD61-FITC fluorescence intensity of single neutrophils with attached platelets (C), and platelet P-selectin (CD62P) expression after 14 minutes of shear (D). Values are mean ± SEM for 4 to 5 experiments using blood from different donors. *$P<0.05$, **$P<0.01$. 
presence of mCRP, antibody blocking also revealed the involvement of P-selectin and Mac-1 in adhesive interactions. Anti–L-selectin mAb produced little or no further inhibition of native CRP or mCRP responses, consistent with CRP- and mCRP-induced L-selectin shedding from neutrophils.\textsuperscript{16,26} Unlike native CRP, mCRP can directly upregulate neutrophil CD11b/CD18 expression.\textsuperscript{26} This action might have contributed to the enhanced formation of neutrophil aggregates, because CD18 blockade resulted in significantly higher decreases in the number of neutrophil aggregates in blood subjected to shear in the presence than the absence of mCRP. It should be noted that adhesion molecules that mediate neutrophil adhesion to immobilized platelets under higher shear levels may differ from those observed in the present study.

Our results point toward the involvement of different Fcγ receptors in native CRP and mCRP signaling. Thus, the actions of native CRP appear to be mediated predominantly through the low-affinity IgG FcγRIIa (CD32), whereas the actions of mCRP are mediated predominantly via the low-affinity immune complex binding IgG receptor FcγRIIIb (CD16). These observations are consistent with previous studies identifying CD32 as the primary binding site for native CRP on leukocytes\textsuperscript{32,33} and with functional studies on mCRP suppression of neutrophil apoptosis.\textsuperscript{25} However, the anti-CD16 mAb did not produce complete reversal of the mCRP actions, raising the possibility that mCRP might activate other as yet unidentified cell surface receptor(s), as suggested by studies on the mCRP activation of endothelial cells.\textsuperscript{43} Clearly, additional studies on isolated cells are needed to identify the additional binding site(s).

Limitations of this study are that the mechanisms regulating mCRP formation from native CRP in vivo and the relevance of a soluble form of mCRP compared with the low-solubility, tissue-associated form\textsuperscript{27} are still unknown. It is unknown whether CRP produced locally in the vessel wall\textsuperscript{44} could be responsible for mCRP that is naturally expressed in the intima.\textsuperscript{27} An endothelial injury may result in exposure of mCRP to blood components, leading to platelet activation and platelet-neutrophil interactions as described here and elsewhere.\textsuperscript{25,26} Alternatively, inflammation may lead to de novo formation of mCRP from native CRP within the blood stream, thereby linking thrombosis and inflammation, 2 of the key events in ACS and recurrent disease after coronary angioplasty.\textsuperscript{9,11} Such actions of mCRP could be aggravated when normal endothelial homeostatic mechanisms, such as nitric oxide and prostacyclin, are compromised. A limitation of flow cytometry analysis is that the neutrophil gate is set using unshered blood and cannot be readjusted during the kinetic measurements. Some monocytes might have been “trapped” in this gate, because shear may also promote formation of neutrophil-monocyte conjugates (with or without platelets)\textsuperscript{34} and might have been mistakenly counted as “neutrophils.” However, considering the ratio of monocytes and neutrophils in normal blood, it is unlikely that this might have had a significant influence on the results.

In summary, our data indicate that although native CRP at clinically relevant concentrations inhibits platelet activation and prevents platelet capture of neutrophils, conformationally altered forms of CRP, such as mCRP, display potent prothrombotic and proinflammatory activities under low levels of shear. Thus, native CRP and mCRP play an opposite role in the regulation of platelet activation and subsequent neutrophil–platelet and neutrophil-neutrophil adhesion, which occur in thrombotic and inflammatory disorders.

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**Disclosure**

Dr Potempa is an inventor on more than 50 patents that involve modified forms of C-reactive protein. He was employed by NextEra Therapeutics, Inc, of Barrington, Ill, and is currently employed by Immtech International, Inc, of Vernon Hills, Ill. NextEra Therapeutics and Immtech International are biotechnology companies that (in aggregate) are the primary owners of intellectual property developed on the modified C-reactive protein from Immtech (until 1999) and from NextEra (from 1998 to 2002). He no longer has a relationship with NextEra Therapeutics.

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