Endothelial C-reactive protein increases platelet adhesion under flow conditions

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Grad E, Pachino RM, Danenberg HD. Endothelial C-reactive protein increases platelet adhesion under flow conditions. Am J Physiol Heart Circ Physiol 301: H730–H736, 2011. First published June 17, 2011; doi:10.1152/ajpheart.00067.2011.—While data regarding the pathogenetic role of C-reactive protein (CRP) in atherothrombosis are accumulating, it is still controversial whether local CRP secretion is of any pathobiological significance. The present study examined whether endothelial-derived CRP modulates autocrine prothrombotic activity. Endothelial cells were isolated from hearts of mice transgenic to human CRP and grown in primary cultures. Human CRP expression was confirmed in these cells compared with no expression in cultures derived from wild-type congenes. Adhesion of human platelets to endothelial cells was studied in the “cone and plate” flow system. Platelet adhesion to cells expressing CRP was significantly increased compared with that in controls (n = 6, P < 0.01). The proadhesive effect of CRP was significantly suppressed in mouse heart endothelial cells and in human umbilical vein endothelial cells following treatment with small interfering RNA for human CRP. Adhesion was modulated by an increase in P-selectin. P-selectin expression correlated with a proadhesive phenotype, and blocking P-selectin with neutralizing antibody significantly decreased the adhesion of platelets to CRP-expressing cells (40.4 ± 10.5 to 9.4 ± 6.9 platelets/high-power field, n = 5 to 6, P < 0.01). In conclusion, human CRP that is locally produced in endothelial cells increases platelet adhesion to endothelial cells under normal shear flow conditions. These findings indicate that CRP exerts a local effect on endothelial cells via P-selectin expression, which promotes platelet adhesion and subsequent thrombus formation.

THE ACUTE PHASE reactant C-reactive protein (CRP) is a strong predictor of cardiovascular morbidity and mortality (27). While initially considered a mere predictor for disease, emerging experimental data support a role for CRP in the pathogenesis of vascular disease. A prothrombotic activity of CRP was reported in animal models (4) and in humans (1). Several mechanisms may be implicated in CRP-induced thrombosis including increased expression of ICAM-1, VCAM-1, and E-selectin adhesion molecules in human endothelial cells (21, 37); induction of monocyte chemoattractant-protein-1 expression in endothelial cells (20) and monocytes (10); induction of plasminogen activator inhibitor-1 expression and activity in human aortic endothelial cells (6) via upregulation of NF-κB activity; and downregulation of endothelial nitric oxide synthase and nitric oxide bioactivity (9).

Earlier perception that CRP is exclusively produced by the liver was recently proven imprecise. CRP production was observed in human aortic endothelial cells (36), the kidney (12), neurons (40), and arterial plaque tissue (16). Serum and carotid plaque CRP levels were found to correlate with the intima-to-media ratio, further suggesting the association of CRP and atherothrombosis disease (38).

While data regarding the pathogenetic role of CRP are accumulating, it is still controversial whether local CRP secretion (29) is of any pathobiological significance. Pepys has questioned whether the existence of CRP at the atherosclerotic plaque area is not necessarily proof of the involvement in the atherothrombotic process (30), emphasizing that “CRP may be an innocent bystander, a victim or possibly an atheroprotective agent” (23). The present study examined whether endothelial production of CRP modulates an autocrine prothrombotic activity.

MATERIALS AND METHODS

Mice. The protocol for this study was approved by the Institutional Animal Care and Use Committee. C57BL/6 congenic CRP (CRPtg) mice carry a 31-kb Crl fragment of human genomic DNA consisting of the CRP gene, 17 kb of 5-flanking sequence, and 11.3 kb of 3-flanking sequence (31). CRP expression in these mice is linked to a testosterone promoter. Thus only male CRPtg mice and male C57BL/6 wild-type mice were used in this study.

Isolation and culture of murine heart endothelial cells. The method for isolation and purification of endothelial cells was modified from a previously published protocol (17). In brief, sheep anti-rat Dynal beads (Invitrogen) were coated with either anti-PECAM-1 or anti-ICAM-2 (Becton Dickinson Labware) monoclonal antibody (1.5 μg antibody for 1 × 10^7 beads) according to the manufacturer’s instructions. Three hearts from 8–12-wk CRPtg or wild-type mice were aseptically harvested and placed in 4°C cold digestion medium (DMEM supplemented with 10% FBS), 12.5 U/ml nystatin, 100 μg/ml streptomycin, and 100 U/ml penicillin (all purchased from Biological Industries). The hearts were finely minced with scissors and digested in 10 ml of type I collagenase (180–200 U/ml in PBS, Worthington) at 37°C for 45 min. The digested tissue was mechanically dissociated by triturating with 14-gauge 6-in. needle connected to a 10-ml syringe, filtered through a 100-μm disposable cell strainer (Becton Dickinson Labware), and centrifuged (1,000 × g, 10 min, 4°C). The cell pellet was resuspended in cold 0.1% BSA in PBS and incubated with 50 μl PECA1-coated beads (10 min, 4°C). The beads were magnetically recovered, and the cells were plated in fibronectin (Biological Industries)-coated flasks and suspended in culture medium (DMEM) containing 20% FBS, 1% l-glutamine, 12.5 U/ml nystatin, 100 μg/ml streptomycin, 100 U/ml penicillin (all from Biological Industries), 1% arachidonic acid (Sigma), 100 μg/ml heparin (20 U/ml; Sigma), and 100 μg/ml endothelial cells growth supplement (BD Biosciences). After the cells reached 70–80% confluence (~5–9 days), the cells were detached using warm trypsin, resuspended in 0.1% BSA-PBS, and incubated with ICAM-2-coated beads (10 min, 4°C). Bead-bound cells were washed and plated in culture medium and passaged further at 1:3 ratio. Confluent endothelial monolayers of multiple preparations were used at passage 2 to 3 for this study.
Parity of the culture. The purity of the murine endothelial cell culture was confirmed using immunofluorescence staining technique with antibodies specific for endothelial cells: CD 102 (ICAM-2) and CD 31 (PECAM-1) (1:50, Becton Dickinson Labware) and secondary donkey anti-rat rhodamine-conjugated antibody (1:200, Jackson ImmunoResearch). Non-endothelial cells were detected using antibody for smooth muscle cell actin conjugated to FITC (1:50, Sigma). Using these methods we verified that the primary MHEC culture contained 85–95% endothelial cells.

Western blot analysis. Total protein was extracted from ~1 × 10⁶ cells after a 24-h incubation with no additives (control), 5 μg/ml LPS (Sigma), small interfering RNA (siRNA) for human CRP, nonactive siRNA, or negative siRNA. Protein extraction was performed using 100 μl of Cell Lytic solution (Sigma) supplemented with 1 μl protease inhibitor cocktail (Sigma). After the quantification of protein concentration using the Bradford method, extracts (20 μg) were loaded onto 5–15% gradient SDS polyacrylamide gel. Transferred proteins were blotted overnight for human CRP (ICL, OR), anti-mouse P-selectin (R&D systems), von Willebrand factor (Abcam, UK), CD 102 (ICAM-2), and purified anti-mouse CD 31 (PECAM-1) (Becton Dickinson Labware). Protein expression was normalized to actin (1:4,000, MP Biomedical) because of the latter constitutive expression. X-ray bands were quantified by scanning densitometry (Tina analysis software). We calculated the expression of the proteins in relation to that of actin in the same sample.

Quantitative real-time PCR. Total RNA was extracted from ~1 × 10⁶ cells after 24 h incubation with no additives (control), 5 μg/ml LPS (Sigma), siRNA for human CRP, or negative siRNA. The extraction was performed using High-pure RNA isolation kit (Roche, Germany), according to the manufacturer’s instructions. First-strand cDNAs were synthesized from 1 μg of RNA using Moloney murine leukemia virus reverse transcriptase (Promega) and random primers (Promega). Real-time PCRs were performed with an ABI prism 7900 (Applied Biosystems). Human CRP, P-selectin, and actin (endogenous control) expression were quantified by SYBR Green PCR. Primers were as follows: mouse actin forward (F)-GGC TGT ATT CCC CTC CAT CG and reverse (R)-CCA GTT GGT AAC AAT GCC ATG, mouse P-selectin F-CAT TCT GGA TCA GTG CTT TGA and R-ACC CCG GAG TTA TTC CAT GGT, and human CRP F-TTT TCG TAT TGT GAC GAC C TAC G and R-TGT GAC GAG TTA TTC CAT GGT. Protein expression was normalized to 18S (a universal internal standard for P-selectin and CRP protein expression. The negative control was designed by Invitrogen to be of the same chemical structure as the target RNAi molecules with no homology to any known vertebrate gene. The negative control siRNA did not change the mRNA levels for human CRP in our CRPtg-derived MHECs. siRNA sequences or negative were transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. All the siRNA transfections were performed 24 h before adhesion assay. Western blot analysis, or real-time PCR experiments.

Human CRP flow cytometry. Human CRP levels were measured in HUVECs after 24 h incubation with negative control or siRNA/CRP. Cells were detached using nonenzymatic cell dissociation solution (Sigma) and stained for 30 min with human FITC-conjugated CRP antibody (5 μg/l × 106 cells, Bethyl) or its matching isotype control (goat IgG control, R&D Systems). Positive cells were measured using FACS DIVA flow cytometer (BD Biosciences).

Platelet adhesion to MHECs from CRPtg condition medium of 1 μg/ml LPS or 100 μg/ml RB40.34 from BD Biosciences) or its matching isotype control antibody (10 μg/ml RB40.34 from BD Biosciences) or its matching isotype control antibody (5 μg/ml RB40.34 from BD Biosciences) or its matching isotype control antibody (5 μg/ml LPS, Sigma), siRNA/CRP, or negative siRNA control. In the neutralizing experiments, P-selectin blocking antibody (10 μg/ml RB40.34 from BD Biosciences) or its matching isotype control (10 μg/ml rat IgG1, Biolegends) was added 1 h before the adhesion study was performed and then was washed out with PBS. Immediately before exposure to shear stress in the CPA (300 rpm for 2 min), the medium was removed and replaced with 180 μl of reconstituted whole blood containing calcine-labeled platelets. The cone and plate apparatus induces laminar flow with uniform shear stress over the entire plate surface covered by the rotating cone. After CPA, the platelets were washed three times with PBS (Biological Industries). Subsequently, 10 arbitrary chosen high-power fields (HPF, ×100) were viewed and photographed using fluorescent microscopy. While the adhesion assays were performed, two pictures were taken from each microscopic field: one of phase contrast for endothelial morphology and the other with FITC labeling for platelets. Using this technique we explored the phase-contrast field for nonendothelial cells. Fields that were contaminated by smooth muscle cells were excluded from FITC-labeling analysis for platelet adhesion. Each experiment was repeated 3–8 times, and the number of the adherent platelet in each field was calculated.

siRNA transfection. Cells were harvested, transferred onto a four-wells plate (adhesion assay) or 25-cm² flasks (Western blot or real-time PCR experiments), and grown in culture medium without antibiotics until they reached 60 to 70% confluence. The stealth small interference RNAs (siRNA) against human CRP and stealth RNAi negative control duplexes were designed by Invitrogen. In a preliminary experiment, three different sequences of siRNA were analyzed and one siRNA-annealed oligonucleotide duplex for human CRP, which reduced the human CRP mRNA after a 24-h incubation, in 60% of our CRPtg-derived MHECs was chosen for all the siRNA experiments. Sequence 5′–3′ sense, GGU CUA AGG AGU GAU ACA GUU U; antisense, AAA CUG UAU CCU AUA UCC UUA GAC C. A second siRNA, which did not change the human CRP mRNA after a 24-h incubation (nonactive siRNA), served as an internal standard for P-selectin and CRP protein expression. The negative control was designed by Invitrogen to be of the same chemical structure as the target RNAi molecules with no homology to any known vertebrate gene. The negative control siRNA did not change the mRNA levels for human CRP in our CRPtg-derived MHECs. siRNA sequences or negative were transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. All the siRNA transfections were performed 24 h before adhesion assay, Western blot analysis, or real-time PCR experiments.

Statistics. Values are means ± SD. Student’s t-test and ANOVA with a Newman-Keuls post hoc test were used to assess differences between two and more than two groups, respectively. Differences were considered significant at P < 0.05.
RESULTS

**MHECs derived from CRPtg mice express human CRP.** Real-time PCR analysis for human CRP show that MHECs derived from human CRPtg mice express mRNA for human CRP, whereas control wild-type MHECs do not. Western blot analysis confirmed the expression of human CRP by CRPtg MHECs, with a light band in wild-types, probably the result of cross-reactivity with murine CRP, based on the lack of human CRP mRNA expression in wild-type-derived endothelial cells while markedly detected in human CRPtg-derived endothelial cells. Incubation with LPS (24 h) further augmented CRP expression, with no effect on its expression in wild-types (Fig. 1).

Platelet adhesion to MHECs is increased in CRPtg-derived MHECs. Platelet adhesion to endothelial cells was measured using calcein-labeled human platelets under physiological arterial shear-stress conditions using the cone and plate device. Platelet adhesion to CRPtg-derived endothelial cells was significantly augmented compared with that of cells derived from wild-type mice (24.3 ± 8.5 vs. 6.3 ± 3.8 platelets/HPF, respectively, n = 6, Fig. 2, A–E, P < 0.01). The proadhesive effect of CRP was significantly increased after treatment with LPS (from 24.3 ± 8.5 to 40.4 ± 10.5 platelets/HPF, n = 6,

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**Fig. 1.** Endothelial cells derived from human C-reactive protein (hCRP) transgenic (tg) mice express hCRP. Total protein was extracted from ~1 × 10³ mouse heart endothelial cells derived from hearts of wild-type (WT) and CRPtg mice. Representative blot images of Western blot analysis performed using antibody for hCRP and actin after incubation with or without LPS. The experiment was repeated 3 times. Blots for actin and CRP were performed on separate gels using the same samples.

**Fig. 2.** Platelet adhesion is augmented in endothelial cells derived from CRPtg mice. Murine heart endothelial cells (MHECs) derived from WT or CRPtg mice were grown in 4-well plates. Cells were incubated for 24 h in control or LPS (5 μg/ml) containing medium. Media was replaced before cone and platelet analysis (CPA; 300 rpm for 2 min) with calcein-labeled platelets. A–D: representative photographs of MHECs derived from WT mice (A and B) and CRPtg mice (C and D) after CPA. Phase contrast photographs (A and C) and fluorescent microscope photographs (B and D) of the same fields are shown. E: platelet adherence after CPA. Data are presented as means ± SD of 6 independent experiments. *P < 0.01, WT vs. CRPtg; **P < 0.01, control vs. LPS.
In contrast, platelet adhesion to wild-type-derived endothelial cells was not affected by LPS (6.3 ± 3.8 and 8.6 ± 4 platelets/HPF, with and without LPS, respectively, n = 6, Fig. 2E). To delineate whether indeed CRP affects endothelial-platelet adhesion, its expression in endothelial cells was blocked with siRNA directed against human CRP mRNA (siRNA/CRP). In a preliminary set of experiments, we tested three different siRNA sequences designed (Invitrogen) against the human CRP gene. Real-time PCR demonstrated that one siRNA sequence decreased CRP message by ~30%, a second siRNA sequence did not change the mRNA for human CRP, and the third siRNA sequence decreased human CRP mRNA by 60% and was thus chosen for further experiments. Treatment with siRNA significantly decreased platelet adhesion to CRPtg-derived endothelial cells compared with control negative siRNA (21.1 ± 6 and 40.45 ± 11.4 platelets/HPF, respectively, n = 3–6, P < 0.01, Fig. 3A). CRP-siRNA had no effect on platelet adhesion to endothelial cells derived from wild-type mice (12.3 ± 5 and 10.1 ± 5.2 platelets/HPF, siRNA/CRP and siRNA negative, respectively). These findings were reproduced in human-derived endothelial cells (HUVECs). Overnight incubation with LPS significantly increased the adhesion of platelets to HUVECs (from 34.7 ± 10 to 76.9 ± 38 platelets/HPF, n = 3–6, P < 0.05, Fig. 3C). Treatment with siRNA/CRP decreased by 60% the expression of human CRP measured in HUVECs using the flow cytometry technique (Fig. 3B) and significantly decreased endothelial-platelet adhesion compared with stealth RNAi negative control (from 34.7 ± 10 to 12.9 ± 6.2 in control and from 76.9 ± 38 to 40.6 ± 19.4 platelets/HPF in LPS treated cells, n = 3–6, P < 0.05, Fig. 3C).

**DISCUSSION**

CRP is present in atherosclerotic lesions, and its levels correlate with the lesion area (30). Most of the circulating CRP is produced in the liver. Several reports confirm that in addition to the liver, CRP is locally expressed in the vasculature, both in normal arteries and in degenerated venous grafts (13). CRP is expressed in vascular smooth muscle cells (3) as well as coronary and aortic endothelial cells (36). There is an ongoing controversy whether the minute amounts of CRP that are locally derived in the vasculature are of any pathobiological significance.

This study used endothelial cells that were derived from transgenic mice that express human CRP. These cells express human CRP and thus make it possible to explore its effects on...
endothelial cells in vitro without the risk of confounding contamination that may occur when using commercial CRP preparations (19, 32). This contained model provides evidence for the physiological importance of those minute amounts of endothelial-derived CRP amounts on endothelial-platelet interactions. The endothelial-expressed CRP increases P-selectin expression, which promotes endothelial adhesiveness to circulating platelets.

In a previous study that used the same cone and plate flow system, we have shown that CRP confers hemostatic phenotype to bovine aortic endothelial cells, promoting platelet adhesion to the vessel wall (39). The CRP used in that study was either purified human recombinant CRP or CRP from sera of transgenic mice. In the present study the endothelial cells are actually a primary culture from the hearts of transgenic mice or from human umbilical vein. Therefore, the CRP here is endogenous and expressed by the endothelium itself. Thus the findings of this study, while supporting the prohemostatic activity of CRP on endothelial cells, convey data as for the autocrine and paracrine activity of CRP in endothelial cells. A putative weakness of our previous report was the use of exogenous CRP and its possible contamination. The present study, which used locally derived CRP as well as its silencing with siRNA, precludes this possibility; while further supporting the conclusion that CRP promotes endothelial-platelet adhesion, it demonstrates that autocrine CRP is responsible to this phenomena and thus maybe of importance in modulating thrombosis.

The interaction between platelets and endothelial cells is a critical step in atherothrombosis that makes the shift toward acute vascular events. This study shows that endothelial-derived CRP upregulates, either directly or indirectly, the endothelial expression of P-selectin, which mediates the tethering and rolling of platelets (18). Platelets are activated when they roll and adhere more tightly to endothelial cells, which in turn become activated. Following their activation, platelets and endothelial cells secrete chemokines, which further generate signals for recruitment of inflammatory cells to the injured site. Inflammation further supports the development and progression of the atherothrombotic process and the subsequent vascular occlusion. Previous studies demonstrated different and contradicting effects of CRP on platelet activation. The reason for this may be the different study conditions and distinct CRP structures that were used for study. The interaction between platelets and neutrophils was enhanced by murine CRP (5) and inhibited by pentameric CRP (14). Fiedel and Gewurz (7) have shown that CRP inhibits platelet activity only after proteolysis. Furthermore, pentameric CRP at levels found during episodes of inflammation directly binds to the activated form of αIIbβ3 and inhibits platelet aggregation (2). In contrast, murine CRP and heat agglutinated CRP have been shown to activate platelets (8, 25). In the current study, CRP was produced locally in its native form and increased the adhesion of platelets to endothelial cells by upregulating P-selectin expression and sP-selectin secretion. Treatment with siRNA directed against human CRP decreased CRP and P-selectin expression, whereas nonactive siRNA did not change CRP or P-selectin expression (Fig. 4D). In addition, treatment with siRNA against human CRP or neutralizing P-selectin using specific antibody significantly reduced the platelet adherence to MHECs. These results imply that there is a direct relationship between CRP and P-selectin.

The evolution of the role of CRP in atherothrombosis deserves a special comment. CRP was found to be a strong predictor for cardiovascular disease almost 10 years ago, based on several monumental studies (27, 28, 34). At that time it was
endothelial cells, in this case via a mediator leading to increased thrombogenicity, CRP is involved in the pathology of atherothrombotic plaque formation. This adhesion is mediated by endothelial-platelet adhesion. This adhesion is mediated by the CRP-induced proadhesive effect and promotes thrombosis. The CRP-induced proadhesive effect and promotes thrombosis. This adhesion is mediated by the CRP-induced proadhesive effect.

Furthermore, CRP was found to be a valid target in reducing cardiovascular morbidity in an acute myocardial infarction model (24). Thus it is quite confident to define CRP as an active vascular modulator, and the current questions now are actually the mechanisms of action and their relative importance in disease modulation. The present study emphasizes the role of locally expressed CRP in thrombus formation with its current clinical sequelae. Circulating CRP, which is measured by routine clinical tests, is mainly produced by the liver. Locally expressed CRP, which is not measured by available tests, may be a separate risk factor as well as a target for therapy. Further studies should verify the association between systemic and locally expressed CRP and whether similar therapy affects both equally.

The effects of CRP on platelet-endothelial adhesion were shown in endothelial cells that were derived from human CRPΔ mice. There are limitations to this animal model including the supraphysiological CRP plasma levels and the fact that serum amyloid protein is the major murine acute-phase reactant rather than CRP. To show that these observations are not limited to transgenic mice alone, we performed another set of experiments. The results produced in these set of experiments suggested increased thrombosis (4) with controversial activity if any on atherogenesis (4, 11, 26). Studies in mice prone to atherosclerosis such as apolipoprotein-E (ApoE) knockout mice (33), ApoE3-Leiden (E3L) transgenic mice (35), or LDL-receptor ApoB100 knockout mice (15) suggest that CRP does not promote the build-up of atherosclerotic plaque. In contrast, CRP was reported to decrease atherosclerosis in mice in one study (15) and accelerate atherosclerosis lesion formation in other experiments (22, 29). The opposing results can be explained by different experimental settings, especially the age of the mice tested (different lesion kinetics develop in older mice) and the knockout type: ApoE, LDL receptor, and ApoE3-Leiden. Epidemiological studies also support the role of CRP in various components of atherothrombosis other than atherosclerosis.

In conclusion, our data demonstrate that human CRP, produced by murine or human endothelial cells, increases platelet adhesion to endothelial cells under normal shear flow conditions. Thus CRP directly modifies the endothelial phenotype and promotes thrombosis. The CRP-induced proadhesive effect was validated by siRNA to human CRP that was shown to decrease CRP mRNA and protein expression and suppressed endothelial-platelet adhesion. This adhesion is mediated by endothelial-derived P-selectin.

The findings support the notion that the presence of CRP at the atherothrombotic plaque area may indicate involvement in the pathology of atherothrombotic plaque formation; although not alone in this region and definitely not the only mediator leading to increased thrombogenicity, CRP exerts a local effect on endothelial cells, in this case via P-selectin expression, which promotes platelet adhesion and subsequent thrombus formation.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

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