C-Reactive Protein Induces Release of Both Endothelial Microparticles and Circulating Endothelial Cells In Vitro and In Vivo: Further Evidence of Endothelial Dysfunction

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BACKGROUND: Inflammation is pivotal in atherosclerosis. A key early event in atherosclerosis is endothelial dysfunction. C-reactive protein (CRP), the prototypic marker of inflammation in humans, is a risk marker for cardiovascular disease, and there is mounting evidence to support its role in atherothrombosis. CRP has been shown to promote endothelial dysfunction both in vitro and in vivo. Emerging biomarkers of endothelial dysfunction include circulating endothelial cells (CECs) and endothelial microparticles (EMPs). However, there is a paucity of data examining the effect of CRP on CEC and EMP production in vitro and in vivo.

METHODS: In this report, we treated human aortic endothelial cells (HAECs) with increasing concentrations of CRP (0–50 μg/mL) or boiled CRP. We counted CECs and EMPs by flow cytometry.

RESULTS: Although CRP treatment resulted in a significant increase in release of both CECs and EMPs, boiled CRP failed to have an effect. Pretreatment of HAECs with sepiapterin or diethylenetriamine NONOate, both of which preserve nitric oxide (NO), resulted in attenuation of CRP’s effects on CECs and EMPs. CD32 and CD64 blocking antibodies but not CD16 antibody or lectin-like oxidized LDL receptor 1 small interfering RNA (LOX-1 siRNA) prevented CRP-induced production of CECs and EMPs. Furthermore, delivery of human CRP to Wistar rats compared with human serum albumin resulted in significantly increased CECs and EMPs, corroborating the in vitro findings.

CONCLUSIONS: We provide novel data that CRP, via NO deficiency, promotes endothelial dysfunction by inducing release of CECs and EMPs, which are biomarkers of endothelial dysfunction.

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C-reactive protein (CRP),2 a member of the pentraxin family, is the prototypic marker of inflammation in humans. CRP is a valid marker of cardiovascular risk, and mounting data support a role for CRP in atherothrombosis (1–4). Several studies have shown a significant relationship between CRP and endothelial dysfunction and that CRP impairs endothelial vaso-reactivity in vivo (5, 6). CRP has been shown to inhibit endothelial nitric oxide synthase (eNOS) activity and bioactivity in vitro and in vivo, resulting in hypertension (7–10). In addition, CRP downregulates endothelial progenitor cell number and function in vitro (11).

Endothelial microparticles (EMPs) are defined as small vesicles (100 nm to 1 mm in diameter) released from endothelial cells in response to activation or apoptosis by various stimuli (12), with a subset of important membrane proteins and phospholipids from their parent cells. Recent studies have demonstrated that EMPs can be used as a novel marker of endothelial injury (13). Accumulating evidence shows that circulating EMP levels are significantly increased in conditions associated with endothelial dysfunction, such as hypertension, dyslipidemia, diabetes mellitus, coronary artery disease, and heart failure (14–18). Interestingly, endothelial NOS uncoupling contributes to EMP release (19), and CRP has been shown to promote eNOS uncoupling (7–10). Hence, in this study, we examined the effect of CRP on EMPs in vitro and in vivo.

Another emerging biomarker of endothelial dysfunction is the quantification of ex-luminal circulating

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2 Nonstandard abbreviations: CRP, C-reactive protein; eNOS, endothelial nitric oxide synthase; EMP, endothelial microparticle; CEC, circulating endothelial cell; LPS, lipopolysaccharide; TLR4, Toll-like receptor 4; siRNA, small interfering RNA; HAEC, human aortic endothelial cell; BH4, tetrahydrobiopterin; DETA, diethylenetriamine; LOX-1, lectin-like oxidized LDL receptor 1; HuSA, human serum albumin; ACS, acute coronary syndrome.

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endothelial cells (CECs). Their presence indicates shedding of the damaged intimal endothelial layer into the blood, resulting in small numbers of these cells in health and increasing numbers in disease states that induce endothelial dysfunction such as diabetes and hypertension (20–22). Increased numbers of CECs are present in other systemic inflammatory diseases, such as lupus and other vasculitides (23, 24). A high CEC level in the setting of acute coronary syndrome, for instance, has been shown to identify individuals at higher risk for subsequent cardiovascular events (25). However, there is a paucity of data examining the effects of CRP on CECs.

Hence, in this study, we examined the effect of CRP on both EMPs and CECs.

Methods

CRP purified from human ascitic or pleural fluids was passed through a Detoxigel column to remove endotoxin [lipopolysaccharide (LPS)] and dialyzed extensively to remove azide as described (5, 7, 26). Our CRP preparations have been shown to have proinflammatory effects in Toll-like receptor 4 (TLR4) small interfering RNA (siRNA) knockdown cells, whereas LPS loses its effect in these cells (26–28). Boiled CRP was also used as control (5, 7, 26).

Cell culture

We used human aortic endothelial cells (HAECs) between passages 3 and 6 as described (5, 7). HAECs were incubated overnight with different concentrations of CRP (0, 12.5, 25, and 50 μg/mL) or boiled CRP (25 μg/mL). For preparation of EMPs, culture supernatants were collected and cleared from cell fragments by centrifugation at 4300g for 5 min. The supernatant was then ultracentrifuged at 200 000g for 120 min at 10 °C. Pelleted EMPs were resuspended in 400 μL PBS (pH 7.6, filtered) and used immediately for flow cytometry.

Flow cytometry for EMPs

We incubated 10 μL EMP suspension with 10 μL anti-human/rat E-selectin, CD144, CD31, and CD42 or isotype control. We labeled another set of cells with antibodies to Annexin V, CD51, CD61, and CD42. After labeling, samples were analyzed by flow cytometry (29). For the EMP measurements, the flow cytometer was kept running for 30 min until the background events were <1%. Microparticles present in PBS were analyzed regarding size and fluorescence. Briefly, on a log forward scatter/log side scatter dot plot, we defined the upper size limit of the microparticles using 1-μm calibrant beads and drew a gate around the population. Only events included in this gate were further analyzed for their fluorescence. We collected 5000 events for each sample, expressed as CD144 +, CD31 +, E-selectin +, and CD42– or Annexin +, CD51 +, CD61 +, and CD42– per 5000 cells.

Flow cytometry for CECs

The flow cytometry protocol was based on a mononuclear cell analysis for size, nuclear complexity, and binding of specific antibodies conjugated to given fluorochromes. After excluding cell debris with a morphological gate, we stained cells with fluorescent-tagged antibodies to CD34, CD146, and CD105 and matched isotype controls. These cells in a subset were found to be negative for CD45 (to rule out lymphocytes).

Mechanistic insights

Because CRP has been shown to decrease eNOS activity by uncoupling (5, 7), we tested the effect of pretreatment with sepiapterin [a tetrahydrobiopterin (BH4) precursor, 100 μmol/L] and diethylenetriamine (DETA) NONOate (nitric oxide donor, 100 μmol/L) for 2 h on CRP-induced CECs and EMPs. Also, because CRP appears to mediate its effects in HAECs via CD32 and CD64, we pretreated HAECs with blocking antibodies to CD16, CD32, and CD64 before treatment with CRP. Finally, because data suggest that CRP binds to lectin-like oxidized LDL receptor 1 (LOX-1) (28), we examined the effect of LOX-1 siRNA on CRP’s effects on EMPs and CECs. Cells were transfected with scrambled or LOX siRNA before addition of CRP.

In vivo effects of CRP in rat CECs and EMPs

Wistar rats were injected with either human CRP (20 mg/kg body weight) or vehicle control [human serum albumin (HuSA)] (n = 4/group) for 3 days as described (27, 28). We obtained blood for CEC and EMP quantification using rat-specific fluorochrome-labeled antibodies followed by flow cytometry, using isotype controls.

Statistical analysis

We used the Student paired t-test for normally distributed data and Wilcoxon signed rank test using Graph Pad Prizm software if data were nonnormal. ANOVA followed by appropriate multiple-comparisons posttest was carried out for experiments having >2 experimental groups. Data are represented as mean (SD), and P < 0.05 was considered statistically significant. All experiments were performed at least 3 times in duplicate.

Results

CRP treatment dose-dependently increased the number of CECs, with significant increase at concentrations >25 μg/mL (ANOVA, P for trend = 0.012) (Fig. 1A).
CRP treatment of HAEcs resulted in increased EMPs, with a dose of 25 μg/mL (P = 0.066) and a significant increase with a CRP concentration of 50 μg/mL (P = 0.033) (Fig. 1B). Whereas we show increased positivity for CD144, CD31, and E-selectin (to phenotypically represent EMPs), we have also examined positivity of Annexin, CD51, and CD61 (markers expressed on EMPs) and obtained results similar to those seen in Fig. 1B. Addition of boiled CRP had no effect on either biomarker of endothelial function, showing that these are effects due to CRP, per se.

We then examined if these effects of CRP were observed in our in vivo model. We have previously shown that human CRP injection into rats resulted in decreased eNOS due to uncoupling, resulting in impaired vasoreactivity (5). Here, we show that injection of human CRP, compared to HuSA, in vivo in rats significantly increased the number of circulating CECs (Fig. 2A) and EMPs (Fig. 2B). We have previously reported that with this system we achieve a plasma concentration of human CRP of around 20 μg/mL (5, 26, 27).

To obtain mechanistic insights, we tested the effect of sepiapterin and DETA NONOate on CRP-induced CECs and EMPs as well as blocking antibodies to Fcγ receptors (CD16, CD32, and CD64). As shown in Fig. 3A, 25 μg/mL CRP significantly increased CEC production, and this was significantly attenuated with sepiapterin and DETA NONOate and antibodies to CD32 and CD64 but not CD16. Similar effects were seen for EMPs. CRP (50 μg/mL) significantly increased EMP number, and this was significantly decreased by pretreatment with sepiapterin and DETA NONOate and antibodies to CD32 and CD64 but not CD16 (Fig. 3B). Transfection of cells with LOX1 siRNA failed to abrogate the effects of CRP (data not shown).

**Discussion**

It is becoming abundantly evident that CRP has a clear role in atherothrombosis, and most effects reported for CRP (azide-free and without endotoxin contamination) appear to bear a relationship to endothelial dys-
function and activation and the polarization of macrophages to a proinflammatory phenotype (2, 27). We and others have shown that CRP impairs endothelial vasoreactivity, via decreased eNOS activity and bioactivity, mediated by eNOS uncoupling (4–10). The initial reports in regard to CRP inhibition of eNOS activity and bioactivity raised the concern that CRP, by inducing endothelial dysfunction, could put patients at risk for hypertension and cardiovascular disease. Recently, this has been confirmed in vivo in rat models by at least 2 groups (10, 30, 31). Thus, additional biomarkers for assessing endothelial dysfunction have now emerged and include CECs and EMPs. In this report, we show for the first time, in vitro and in vivo, that CRP promotes significantly increased release of CECs and EMPs from aortic endothelial cells.

One of the key early events in atherosclerosis is endothelial activation and dysfunction. During sustained endothelial cell activation, endothelial cells may physically detach from the vessel wall by a process involving the initiation of proapoptotic signaling cascades and loss of cell–cell contact and anchoring proteins. These detached endothelial cells, referred to as circulating endothelial cells, can be used as a marker of severe vessel wall damage. CECs have been shown to be increased in acute coronary syndrome (ACS) and in diabetes and heart failure, where CRP concentrations are also increased (20–25). In this report, we show that CRP promotes CEC release from aortic endothelial cells in vitro and in rats in vivo, further supporting the data that endothelial dysfunction is induced by CRP.

With regard to the effect of CRP on EMPs, Wang et al. (19) showed that in human umbilical vein endothelial cells, CRP treatment in vitro resulted in increased EMP generation, and this was associated with a downregulation of NO production secondary to decreased bioavailability of BH4 in endothelial cells. The purity of their CRP preparation was not reported, and the fact that they saw effects at lower concentrations than in the present report (20 vs 50 μg/mL) raises the issue of contribution of contamination such as endotoxins. In this study, we have gone further and confirmed these findings in HAECs (the primary site of atherosclerosis) and also reported on the increase in CECs. In addition, we have confirmed these findings in vivo. Because we have observed increased E-selectin as well as increased Annexin staining, we believe that these EMPs are biomarkers of endothelial activation and apoptosis.

We have previously shown that addition of sepiapterin, a BH4 precursor, reverses CRP inhibition of eNOS (7). In this study, a BH4 precursor and an NO donor, DETA NONOate, had similar effects, i.e., they were able to attenuate the effects of CRP on numbers of both CECs and EMPs. Thus, impaired eNOS activity appears to be proximal to these events, since rescuing eNOS from uncoupling and replenishing NO abrogates those effects. Also, CRP’s biological effects in endothelial cells appear to be mediated via the Fcγ receptors, CD32 and CD64 (32). In this investigation, we also showed that CRP’s effects in inducing EMPs and CECs is abrogated with antibodies to CD32 and CD64 but not CD16. These mechanistic pathways by which CRP exerts its effects on EMPs and CECs need to be confirmed in vivo in future studies.

In conclusion, this report of our study adds to the published literature demonstrating that CRP induces endothelial dysfunction by reporting on novel additional biomarkers, CECs and EMPs. These effects appear to be mediated by Fcγ receptors, CD32 and CD64, and are due to NO deficiency induced by CRP. These events could result in pronounced endothelial dysfunction in vivo and promote clinical events such as hypertension and cardiovascular disease.

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**Fig. 3.** Mechanisms by which CRP upregulates CECs (A) and EMPs (B). Cells were pretreated with sepiapterin (Sepi), DETA NONOate, or antibodies to CD16, CD32, or CD64 and then incubated with increasing concentrations of purified human CRP (0, 12.5, 25, 50 μg/mL) or boiled CRP (50 μg/mL) for 24 h. Numbers of CECs and EMPs were assessed by flow cytometry. *P < 0.05 compared to controls; #P < 0.05 compared to CRP 25 and/or 50 μg/mL. n = 4 experiments. Ab, antibody.
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References