C-Reactive Protein Does Not Directly Induce Tissue Factor in Human Monocytes

Elaine Paffen, Hans L. Vos, Rogier M. Bertina

Objective—It is generally assumed that C-reactive protein (CRP) induces synthesis of tissue factor (TF) in monocytic cells, thereby potentially initiating intravascular blood coagulation. We aimed to elucidate the mechanism of CRP-induced TF expression in monocytes and monocyte-derived macrophages (MDMs) in vitro.

Methods and Results—Monocytes were isolated from the blood of healthy donors and cultured with or without CRP or lipopolysaccharide (LPS) to study the time course of TF antigen and TF mRNA expression. Addition of 100 μg/mL CRP did not result in a significant increase in TF antigen (range: 9 to 163 pg/10⁶ cells, n=11) and TF mRNA (relative number of TF transcripts; Nₜₐₛ=0.01 to 0.33), when compared with nonstimulated cells (TF antigen 7 to 46 pg/10⁶ cells, Nₜₐₛ=0.01 to 0.13). Variation of CRP concentration and exposure time did not affect the TF response. Similar results were obtained in monocytes cultured in suspension and in MDMs. In contrast, TF was strongly induced by 10 μg/mL LPS (TF antigen 1125 to 6120 pg/10⁶ cells, Nₜₐₛ=5.94 to 23.43). Cultured monocytes did express FcRγII, a putative CRP receptor, and addition of CRP induced a 7-fold increase in the production of monocyte chemoattractant protein-1 (MCP-1). Interestingly, CRP addition to peripheral blood mononuclear cells (PBMCs) did result in TF expression on monocytic cells.

Conclusions—The absence of TF induction after incubation of purified monocytes with CRP indicates that CRP is unable to induce TF expression in monocytes and MDMs directly. The presence of CRP-induced TF expression in PBMCs suggests that CRP can induce TF indirectly, probably through cross-talk between cells. (Arterioscler Thromb Vasc Biol. 2004;24:1-8.)

Key Words: C-reactive protein ▪ tissue factor ▪ monocytes ▪ MCP-1 ▪ atherosclerosis

Atherosclerosis is the major cause of arterial thrombosis and is generally characterized as an inflammatory disease.1 Peripheral blood monocytes and lymphocytes are recruited to the early atherosclerotic lesion and elicit the inflammatory response. In the arterial intima, monocytes and monocyte-derived macrophages (MDMs) accumulate and express tissue factor.2 TF, a transmembrane glycoprotein, is the primary initiator of the serine protease cascade of the coagulation system.3 Although TF is not constitutively expressed by cells from the vasculature, various agents can induce TF expression in human monocytes. Inflammatory mediators like bacterial endotoxins (lipopolysaccharides; LPS) or phorbol myric acid are potent inducers of TF expression by monocytes.4,5 The mechanism by which TF is induced in the atherosclerotic lesion is not understood, but inducible TF expression has been suggested to play an important role in the pathogenesis of atherosclerosis.6,7

In the arterial intima, the acute phase plasma protein C-reactive protein (CRP), which is a marker for inflammation and a predictor for cardiovascular events,8-10 colocalizes with monocytic cells.11,12 The serum concentration of CRP, a pentraxin synthesized in the liver, increases >1000-fold on inflammation. CRP amplifies and facilitates innate immunity by binding complement factor C1q and activating the classical complement pathway.13,14 It also binds to phosphatidylincholine and participates in the clearance of apoptotic and necrotic cells.15,16 Its role as a procoagulant was first suggested by Whisler,17 who measured increased procoagulant activity (PCA) in monocytic cells after addition of CRP. Later, CRP was reported to induce synthesis of TF in peripheral blood mononuclear cells (PBMCs).18 To date, it is generally assumed that CRP induces TF expression in human monocytes, thus linking inflammation, coagulation, and thrombosis.19-22 Human monocytes express FcRγIIa or CD32, which binds CRP with high affinity and has been described as putative CRP-receptor23 and FcRγI or CD64, which binds CRP with lower affinity.24 However, existence of a third and possibly FcR-independent and still poorly defined CRP-R on monocytes, cannot be excluded.25-28 Given these multiple manners by which CRP interacts with monocytes, we were interested to learn whether CRP can induce TF in isolated monocytes and, if so, via which mechanism. We report here that CRP does not induce TF expression in

Received September 30, 2003; revision accepted March 16, 2004.
From the Hemostasis and Thrombosis Research Centre, Department of Hematology, Leiden University Medical Centre, Leiden, the Netherlands.
Correspondence to Elaine Paffen, Hemostasis and Thrombosis Research Centre, Department of Hematology, Leiden University Medical Centre, Albinusdreef 2, 2333 ZA Leiden, the Netherlands. E-mail epaffen@lumc.nl
© 2004 American Heart Association, Inc.

Arterioscler Thromb Vasc Biol is available at http://www.atvbaha.org
DOI: 10.1161/01.ATV.0000126681.16619.69
monocytes directly, but rather requires other blood cells to mediate its effect.

**Methods**

**Reagents**

Highly purified (>90%) human CRP (#C-4063) and LPS (from *Salmonella typhimurium*) were obtained from Sigma-Aldrich Chemical GmbH (Steinheim, Germany). Recombinant human CRP (rhCRP, from *Escherichia coli*, #236608) and purified CRP (#236603) were derived from Calbiochem (La Jolla, Calif). Only those CRP preparations that contained endotoxin levels <10 pg/mL, as measured in a *limulus amebocyte lysate* assay (Chromogenix AB, Mölndal, Sweden) were used in our experiments. Human fibronectin was purified from plasma. Hepes-buffered RPMI 1640 was supplemented with 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and heat-inactivated (HI) fetal bovine serum (FBS) (Invitrogen Ltd, Paisley, UK) or human AB serum (HSab) (#H-1513, Sigma). Culture medium with supplements, fibronectin, and HSab all were endotoxin-free (<10 pg/mL). Antibodies used in flow cytometry were: phycoerythrin (PE) conjugated anti-CD14 (Becton Dickinson, San Jose, Calif) and anti-CD32 (Ancell Laboratories, and FITC-conjugated antitissue factor (American Diagnostica Inc, Greenwich, Conn), anti-CD14, anti-CD45, and anti-CD71 (Becton Dickinson). Total RNA was isolated using RNaseasy Mini Kit (Qiagen GmbH, Germany). Oligo-(dT) primer and superscript II reverse-transcriptase were from Invitrogen. PCR buffer, 3 mmol/L MgCl₂, 0.2 mmol/L dNTPs, and Taq Hot Goldstar polymerase were provided by the qPCR Core kit, which, together with primers and fluorescent TET/TAMRA-labeled probes, were from Eurogentec (Seraing, Belgium).

**Monocyte Isolation**

Human monocytes were isolated from fresh single-donor buffy coats (Sanquin Bloodbank, NL). PBMCs were isolated by density gradient centrifugation on Ficoll/amidotrizoate (6% Ficoll, 9% NaN₃amidotrizoate, d=1.077 g/mL) and lysis of erythrocytes. From these PBMCs, a highly purified monocyte population (>95% CD14+ positive cells) was obtained by depletion of nonmonocytic cells using magnetic cell sorting technology (MACS) (monocyte isolation kit and Automacs from Miltenyi Biotec GmbH, Bergisch-Gladbach, Germany). Briefly, cells were resuspended in binding buffer (2 mmol/L EDTA, 400 μg/mL human serum albumin, and 1% HSab in PBS) and incubated with hapten-conjugated antibodies against CD3, CD7, CD45RA, CD19, CD56, and IgE for 15 minutes at 4°C in the dark, washed with binding buffer, and incubated with antihapten antibody-conjugated microbeads. The nonlabeled fraction in the dark, washed with binding buffer, and incubated with fluorescent TET/TAMRA-labeled probes, were from Eurogentec (Seraing, Belgium).

**Cell Culture**

Monocytes, 1 to 2×10⁶ cells/mL with a viability >95%, as determined by trypan blue exclusion, were cultured adherent on plastic Petri dishes coated without (Mo-A) or with 50 μg/mL fibronectin (Mo-FA) or in suspension (Mo-S) on Petri dishes with hydrophobic Teflon-FEP film (gauge 25 μm; Janssens NV, St. Niklaas, Belgium) in RPMI 1640 containing 10% HSab HI at 37°C in a humid 5% CO₂ atmosphere. After 2 hours, Mo-FA were washed and fresh medium was added. Stimulation of the cells with CRP or LPS started after 12 hours of culture. PBMCs, 2×10⁶ cells/mL, were cultured on fibronectin-coated Petri dishes with 10% HSab HI.

**Antigen Assays**

Cells were resuspended in extraction buffer (50 mmol/L EDTA, 100 mmol/L NaCl, 1% Triton X-100, pH 7.5) to prepare cell lysates (∼1×10⁷ cells/mL) by 4 cycles of freeze-thawing followed by centrifugation. The supernatant cell extract was used for measuring TF antigen (Elisa 845, American Diagnostica Inc). Results were expressed in pg TF antigen/10⁶ cells using the TF standard included in the kit. Interassay variation was monitored using dilutions of Innovin, a source of human TF, as a standard (300 ng/mL; Baxter Diagnostica Inc, Deerfield, Ill.). In the cell-free supernatant monocyte chemoattractant protein-1 (MCP-1) was measured by Quantakine ELISA (R&D systems, Minneapolis, Minn). Samples were tested in 3 different dilutions in duplicate.

**Flow Cytometry**

Harvested cells were incubated with diluted PE-conjugated and FITC-conjugated antibodies at 4°C for 40 minutes. Afterward, cells were washed in PBS with 0.1% BSA, pelletted by centrifugation, and fixed with 100 μL 1% paraformaldehyde. Fluorescence was analyzed on a FACSscan flow cytometer (Becton Dickinson) counting 10,000 events per sample. Data acquisition and analysis were performed using CellQuest software. Cells were gated using forward light scatter (FSC) versus sideward light scatter (SSC). Surface expression of CD71 and CD32 was described as percentage of CD14+ cells and corrected for nonspecific staining by subtracting the fluorescence of cells stained with a nonspecific isotype antibody.

**Real-Time Polymerase Chain Reaction**

Total RNA (1 μg) was reverse-transcribed with 0.5-μg oligo-(dT) primer using 10 μL superscript II reverse transcriptase in 20 μL RNA reaction buffer. Reverse-transcribed materials were amplified with Taq Hot Goldstar DNA polymerase by adding TET(5′)/TAMRA(3′) fluorescent labeled probe (5′-CCCTGTCGCAAGTACCCTTCCTGA-3′) and sense and antisense primers specific for human TF mRNA (sense primer, 5′-GGACCGAAAACTGTCGTTAAG-3′; antisense primer, 5′-TCCGGAGTCTGTCAGAAGTG-3′) or the TET(5′)/TAMRA(3′) fluorescent-labeled probe (5′-ACCAGCAGAAGTTGCTCCAGAAGTG-3′) and sense and antisense primers specific for human MCP-1 mRNA (sense primer, 5′-CGCTAGGAGCTAGTCCATC-3′; antisense primer, 5′-TTGTCCGACTGGGTCATCTTC-3′). β-Actin cDNA amplification was performed in the same way using (TET(5′)/TAMRA(3′) fluorescent-labeled probe 5′-TGATCCTGCGCATGTCATGTTGC-3′, sense primer, 5′-AGGACCGAAAACTGTCGTTAAG-3′; and antisense primer, 5′-GCTGAGTATAGCAGTACATGG-3′). Quantitative TF mRNA expression was assessed by use of real-time Taqman PCR technology (ABI Prism 7700 Sequence Detection System; Perkin Elmer Applied Biosystems). The PCR reaction consists of a 2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute. The number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold is a measure for the concentration of TF cDNA in the sample. This value for the number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute). The number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute). The number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute). The number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute). The number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (2-step protocol: 10 minutes at 95°C, and 55 cycles amplification at 95°C for 15 seconds, and 60°C for 1 minute).

**Statistical Methods**

Statistical analysis was performed using the paired samples t test using SPSS 11.0 software.

**Results**

**Effect of CRP and LPS on TF Antigen Expression of Purified Human Monocytes in Culture**

Addition of 20 μg/mL CRP to Mo-FA of 3 independent donors did not result in TF antigen expression significantly different from that in unstimulated monocytes. In contrast, addition of LPS to these cells resulted in strong expression of TF-antigen, although the degree of induction varied considerably from donor to donor. Further increasing the CRP concentration to 100 μg/mL did not result in induction of TF antigen (Table 1A). TF antigen in cell lysates of monocytes treated with CRP ranged from 9 to 163 pg/10⁶ cells with a median of 64 pg/10⁶ cells (Table 1A). Unstimulated cells did not express detectable levels of TF antigen.
express similar levels of TF antigen (7 to 46 pg/10⁶ cells, median of 12 pg/10⁶ cells). Stimulation of Mo-FA with 10 μg/mL LPS resulted in strong induction of TF antigen (1125 to 6120 pg/10⁶ cells, median of 3608 pg/10⁶ cells) and TF cell surface expression (55% to 92% TF/CD14 cells, median of 57%). Mo-FA contained 95% CD14 cells and 66% to 100% of the monocytes expressed the putative CRP receptor (FcγRII or CD32; Figure 1A). Variation in the length of the incubation period (1 to 24 hours) emphasized the widely different effects of LPS and CRP on TF expression (Figure 2). TF antigen expression was induced by LPS and peaked at 6 to 8 hours of stimulation, whereas CRP had no effect on TF antigen expression.

Effect of CRP and LPS on TF mRNA Expression of Mo-FA
Because we could not detect TF expression at the protein level, and because CRP has been described to induce de novo TF synthesis,18 we also studied the effect of CRP on TF mRNA levels in cells from the same cultures used for measuring protein expression. Addition of CRP did not result in increased TF mRNA (NTF = 0.01 to 0.33, median NTF = 0.04) compared with unstimulated cells (NTF = 0.01 to 0.13, median NTF = 0.03). In contrast, addition of 10 μg/mL LPS resulted in a 300-fold increase in the level of TF mRNA (NTF = 5.94 to 23.43, median NTF = 9.29) compared with control cells (Table 1A). As expected, LPS-induced TF mRNA

<table>
<thead>
<tr>
<th>TF Ag</th>
<th>TF mRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Range (pg/10⁶ cells)</td>
<td>Median (pg/10⁶ cells)</td>
</tr>
<tr>
<td>A. Control Mo-FA</td>
<td>7–46</td>
</tr>
<tr>
<td>CRP</td>
<td>9–163</td>
</tr>
<tr>
<td>LPS</td>
<td>1125–6120</td>
</tr>
<tr>
<td>B. Control Mo-S</td>
<td>8–123</td>
</tr>
<tr>
<td>CRP</td>
<td>45–166</td>
</tr>
<tr>
<td>LPS</td>
<td>729–3976</td>
</tr>
<tr>
<td>C. Control MDM</td>
<td>24–53</td>
</tr>
<tr>
<td>CRP</td>
<td>78–313</td>
</tr>
<tr>
<td>LPS</td>
<td>496–3151</td>
</tr>
</tbody>
</table>

**Table 1. Induction of TF Antigen and mRNA Expression in Monocytes and MDM by CRP and LPS**

TF antigen (pg/10⁶ cells) expression was measured by ELISA in cell lysates of (A) adherent monocytes (Mo-FA, n=11), (B) monocytes in suspension (Mo-S, n=4), or (C) macrophages (MDM, n=4) after 8 hours of stimulation with 100 μg/mL CRP or 10 μg/mL LPS. From the same cells, RNA was isolated. TF mRNA was amplified by qPCR, related to β-actin mRNA, and expressed as NTF = 2⁻¹⁰Ct.

**Figure 1.** FACS analysis of cell surface expression of Mo-FA or MDMs (A) and PBMCs (B) cultured in presence or absence of 100 μg/mL CRP or 10 μg/mL LPS. A-1, CD32 PE and CD14 FITC expression of control Mo-FA. A-2, CD14 PE and CD71 FITC expression of control MDM. A-3, CD14 PE and TF FITC expression of control Mo-FA. A-4, CD14 PE and TF FITC expression of Mo-FA stimulated with LPS for 8 hours. B-1, CD14 PE and CD45 FITC expression of control PBMCs. B-2, CD32 PE and CD14 FITC expression of control PBMCs. B-3, CD14 PE and TF FITC expression of control PBMCs. B-4, CD14 PE and TF FITC expression of PBMCs stimulated with CRP for 6 hours. The figure summarizes the results of representative experiments.
expression peaked at 4 hours, whereas no CRP-induced TF mRNA expression was observed (Figure 2).

**Influence of Culture Conditions**

Most studies that reported on CRP-induced TF expression in monocytes used cells purified by adherence to plastic culture dishes. However, culturing cells in suspension may represent a more natural state for peripheral blood monocytes and may influence cell susceptibility to a stimulus. We therefore cultured monocytes from the same donor in 3 different ways: adhering to fibronectin-coated Petri dishes (Mo-FA; see Table 1A), adhering to plastic (Mo-A, unpublished data 2003), and in suspension on a Teflon membrane (Mo-S; see Table 1B). Although there were clear morphological differences between these differently cultured monocytes, none of the cultures expressed TF on stimulation by CRP. Only LPS was capable of inducing TF antigen and mRNA expression (Table 1B).

**Effect of CRP and LPS on TF Expression of MDMs**

To assess if differentiated monocytes responded differently to CRP, we investigated the effect of CRP on TF expression of MDMs. During prolongation of the monocyte culture, cells spontaneously differentiate and mature into macrophages in vitro. MDMs were characterized by expression of the macrophage-specific transferrin-receptor (CD71) by FACS. On day 4 of the culture, 95% of the living cells had differentiated into MDMs (Figure 1A). In 4 independent experiments stimulation of MDMs for 8 hours with 100 μg/mL CRP resulted in some TF antigen expression (78 to 313 pg/10^6 cells, median 151 pg/10^6 cells) compared with unstimulated cells (24 to 53 pg/10^6 cells, median 25 pg/10^6 cells), but far less than after stimulation with 10 pg/mL LPS (496 to 3151 pg/10^6 cells, median 1016 pg/10^6 cells; Table 1C). In the same stimulated cells, TF mRNA expression was increased after stimulation with LPS (NTF = 15.35 to 22.64, median 17.89; Table 1C). However, CRP-stimulated MDMs expressed similar levels of TF mRNA (NTF = 0.04 to 0.26, median 0.15) as unstimulated cells (NTF = 0.06 to 0.52, median 0.16; Table 1C). Figure 3 shows the time course of TF mRNA and TF antigen expression after stimulation of MDMs with CRP or LPS. MDMs are extremely sensitive to low concentrations of LPS; even 10 pg/mL, which is close to the detection limit of the limulus

![Figure 2](image2.png)

**Figure 2.** Effect of CRP and LPS on TF antigen (─) and TF mRNA (---) expression of monocytes cultured on fibronectin (Mo-FA); 100 μg/mL CRP (○) or 10 μg/mL LPS (△) were incubated with Mo-FA (control □) for 1 to 24 hours. After stimulation either cell extracts were prepared for TF antigen analysis or RNA was isolated to measure TF mRNA. The figure summarizes the results of a representative experiment.

![Figure 3](image3.png)

**Figure 3.** Effect of CRP and LPS on TF antigen (─) and TF mRNA (---) expression of monocyte-derived macrophages (MDM) cultured on fibronectin; 100 μg/mL CRP (○) or 10 μg/mL LPS (△) were incubated with MDM (control □) for 1 to 24 hours. After stimulation, either cell extracts were prepared for TF antigen analysis or RNA was isolated to measure TF mRNA. The figure summarizes the results of a representative experiment.
amebocyte lysate assay, was able to elicit a significant response.36,37 Because of this extreme sensitivity to LPS, which may lead to spurious TF expression in the CRP experiments, and because of the discrepancy between the mRNA and protein data, we conclude that TF expression in MDMs is not stimulated by CRP.

Functionality of CRP Preparations: Induction of Monocytic MCP-1 Expression

The negative results reported raised the question whether the CRP preparations used have biological activity and whether the monocytic cells have a functional response system toward CRP. We tested this by measuring its effect on the secretion of monococyte chemoattractant protein-1 (MCP-1) in the conditioned medium of cultured monocytes. A synthetic peptide derived from CRP has been found to increase the expression of the chemokine MCP-1 by monocytes,38 and also in human umbilical vein cells, CRP did induce MCP-1 expression.39 Very large differences in MCP-1 expression exist between monocytes from different donors. The average stimulation of MCP-1 production by CRP was 7-fold in Mo-FA from 11 different donors (range: 2.3 to 20.9). Addition of 100 μg/mL CRP during 8 hours resulted in increased MCP-1 antigen (range from 0.28 to 23.45 ng/10^6 cells with a median of 6.92), as well as increased MCP-1 mRNA levels (median N_{MCP-1}=1.83 and mRNA median N_{MCP-1}=107). This indicated a specific biological response of the monocytes to CRP. It proved that the CRP preparations were functional and that the cells have an intact response system toward CRP. Increased MCP-1 expression caused by CRP was similar or even exceeded the effect of LPS on MCP-1 expression. Addition of 10 μg/mL LPS to Mo-FA during 8 hours induced MCP-1 antigen (1.13 to 28.35 ng/10^6 cells with median 6.31) and mRNA expression (median N_{MCP-1}=3040).

Effect of CRP on TF Expression in Cultured PBMCs

So far, we have been unable to demonstrate that CRP induces TF antigen and/or mRNA in highly purified human monocytes in vitro. Previous reports on CRP-induced TF expression have used PBMCs or monocytes purified from PBMCs by adherence, which still contained a significant percentage of nonmonocytic cells.18,31 Therefore, we investigated the effect of CRP on TF expression by monocytes cultured in the presence of other leukocytes. In these experiments, we cultured the complete PBMC preparation, which contains 10% to 30% monocytes as judged by CD14 positivity. In the PBMCs, CD32 was expressed not only by monocytes but also by other cell types (Figure 1B). Interestingly, in these cultures, CRP addition resulted in increased TF expression on the monocytes compared with control cultures (Table 2).

<table>
<thead>
<tr>
<th>TF Antigen (pg/10^6 cells)</th>
<th>Median (pg/10^6 cells)</th>
<th>TF mRNA</th>
<th>Median (N_{TF}-2^10)</th>
<th>Median (N_{TF}-2^15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control PBMC</td>
<td>0–27</td>
<td>4</td>
<td>0.04–0.14</td>
<td>0.07</td>
</tr>
<tr>
<td>CRP</td>
<td>41–543</td>
<td>154</td>
<td>0.61–5.33</td>
<td>2.77</td>
</tr>
<tr>
<td>LPS</td>
<td>499–1701</td>
<td>776</td>
<td>1.12–11.39</td>
<td>4.43</td>
</tr>
</tbody>
</table>

TF antigen (pg/10^6 cells) expression was measured by ELISA in cell lysates of a culture of peripheral mononuclear cells (PBMCs, n=6) after 6 hours of stimulation with 10 μg/mL LPS. From the same cells, mRNA was isolated. TF mRNA was amplified by qPCR, related to β-Actin mRNA, and expressed as N_{TF}=2^ΔCt.

Figure 4. Effect of CRP and LPS on TF antigen (N) (A) and TF mRNA (N) (B) expression of PBMCs cultured on fibronectin. 100 μg/mL CRP (E) or 10 μg/mL LPS (D) were incubated with PBMC (control •) for 1 to 24 hours. After stimulation either cell extracts were prepared for TF antigen analysis or RNA was isolated to measure TF mRNA. The figure summarizes the results of a representative experiment.
Discussion

It has been generally accepted that CRP induces TF expression in monocytic cells.\textsuperscript{17,18} This induction of TF, the initiator of blood coagulation, by the acute phase protein CRP has often been described as one of the mechanisms that link inflammation, coagulation, and thrombosis.\textsuperscript{19–22} In atherosclerotic lesions, CRP is present and TF is expressed by monocytes/macrophages recruited from the blood by the inflammatory signals. On rupture of the unstable plaque, this TF is exposed to the blood, initiates coagulation, and contributes to thrombus formation. Therefore, TF has been proposed to be a key mediator of thrombosis in atherosclerosis.\textsuperscript{5,6} In addition, CRP has been described to be a strong predictor for cardiovascular risk.\textsuperscript{10} Although many reviews refer to CRP-mediated induction of TF in monocytic cells,\textsuperscript{8,9} we were unable to demonstrate CRP-induced TF expression in highly purified human monocytes. We demonstrated that variations in CRP concentrations (20 to 100 $\mu$g/mL) in culture conditions (adherent versus suspension cells) or in incubation time (1 to 24 hours) did not lead to TF expression, neither at the level of TF antigen nor at the level of TF mRNA. When recombinant human CRP was used to stimulate monocytic TF expression, it also seemed to fail in inducing TF expression, but these experiments could not be repeated because of the fact that subsequent preparations all were contaminated with endotoxin (20 to 660 pg/100 $\mu$g CRP).

CRP has different functions: it binds to complement factor C1q, phosphatidylcholine, nuclear constituents, or low-density lipoproteins or very LDL (LDL or VLDL), and CRP binds to an aspecific set of receptors. When CRP binds to C1q, it activates the classical pathway of the complement system.\textsuperscript{13,14} When CRP binds to phosphatidylcholine and LDL, it aids in opsonization and priming for phagocytosis.\textsuperscript{16,40} But it is largely unknown which cellular events are triggered by binding of CRP to a receptor. In our study, we confirmed that CD32, the putative CRP receptor, was expressed on Mo-FA, MDM, and in PBMCs. However, it cannot be excluded that binding of CRP to another CRP-R is needed to evoke a cellular response.\textsuperscript{26,27} Indirect interaction of CRP by binding C1q present on the cell surface of monocytic cells was made unlikely by heat inactivation of the human AB serum used in the culture medium. AB serum also contains VLDL, and VLDL has been described to bind CRP.\textsuperscript{41} However, the VLDL concentration in this serum was so low (0.016 mmol/L) that we can exclude the possibility that VLDL competes away all CRP from the cells.

The effect of CRP on MDMs was similar in dose and time course to that on monocytes. The small increase in TF antigen expression sometimes observed after stimulation with CRP could have been the effect of a very small amount of endotoxin present in the CRP preparations. MDMs are known to be much more sensitive to LPS than monocytes.\textsuperscript{36} Like in monocytes, CRP addition increases MCP-1 production in MDMs (unpublished data, 2003). However, the 2-fold increase in MCP-1 production by MDMs after CRP addition is lower than the induction in Mo-FA, probably because of the higher basal levels of MCP-1 expression by control MDMs. CRP-induced MCP-1 expression in human monocytes and MDMs indicates that the cell are responsive to the CRP preparations used and that the preparations are biologically active.

Our results show that CRP induces MCP-1 antigen and mRNA expression in Mo-FA. MCP-1, or small inducible cytokine A2, is a CC-chemokine chemotactic for human peripheral monocytes. Synthesis of MCP-1 by blood leukocytes has been described to be induced by LPS and cytokines as IL-1$\alpha$.\textsuperscript{42} MCP-1 is present in human atherosclerotic plaques.\textsuperscript{43} CRP-induced MCP-1 expression has been described before,\textsuperscript{39,44} but the mechanism of induced MCP-1 expression in human monocytes is not clear. Interestingly, MCP-1 has been reported to induce TF in monocytes\textsuperscript{45} at doses easily produced in our cell cultures. We could not confirm this finding using isolated monocytes in culture and MCP-1 concentrations up to 20 nM.

Having established that the monocytes were responsive to the CRP preparations and that differences in culture conditions and differentiation state could not account for the absence of TF expression in isolated monocytes, we turned to PBMC cultures, in which the monocytes were cultured in the presence of other leukocytes. Remarkably, we measured increased TF antigen as well as increased TF mRNA expression in PBMCs after CRP addition (Figure 1B and Table 2). This resembles the observations described by Cermak et al.\textsuperscript{18} We did not find that CRP was a stronger inducer of TF expression in PBMCs than LPS, although the levels of induced TF expression in PBMCs varied considerably between different donors. FACS analysis demonstrated that the expressed TF derives from monocytes present in the PBMCs culture. Also, we demonstrated that cultures of purified monocytes do not respond to CRP with increased TF expression. Therefore, it can be concluded that cell–cell interactions or other forms of cross-talk between cells are required for CRP induction of TF in vitro. This is supported by the in vivo observation that elevated serum CRP in patients with various diseases seems to be correlated to TF mRNA expression of leukocytes.\textsuperscript{46} The possibility of CRP being an indirect inducer of TF expression by monocytes has been described earlier. More specifically, it has been suggested that the presence of IFN$\gamma$, secreted by activated T-lymphocytes, modulates TF expression of monocytes.\textsuperscript{30} Preliminary experiments did not reveal any effect of IFN$\gamma$ or CD40 ligand on TF expression in isolated monocytes.

That an acute phase protein might exert modulating effects on TF expression has been recently described by Napoleone et al, who reported that PTX3 enhanced the TF response of human umbilical vein cells stimulated by IL-1$\beta$ and TNF$\alpha$.\textsuperscript{47}

In conclusion, our studies indicate that the induction of TF by CRP is a much more complicated process than initially reported, and further studies are needed to identify the signaling pathways that contribute to this potentially important link between inflammation and thrombosis.

Acknowledgments

The authors thank I. K. van der Linden for technical assistance. This work was supported by a grant of the Netherlands Heart Foundation (NHS 98090).

References

16. Chang MK, Binder C, Torzewski M, Witztum JL. From the Cover: C-reactive protein binds to both oxidized LDL and apoptotic cells through a receptor for C-reactive protein on leukocytes which is fcgamma receptor II. Circulation. 2000;101:1718–1720.
17. Penn MS, Topol EJ. Tissue factor, the emerging link between inflammation, thrombosis, and vascular remodeling. Circ Res. 2001;89:1–2.
C-Reactive Protein Does Not Directly Induce Tissue Factor in Human Monocytes

Elaine Paffen, Hans L. Vos and Rogier M. Bertina

Arterioscler Thromb Vasc Biol. published online March 25, 2004;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2004 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2004/03/25/01.ATV.0000126681.16619.69.citation

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org/subscriptions/