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/10^6 cells, n=11) and TF mRNA (relative number of TF transcripts; N_{TF}=0.01 to 0.33), when compared with nonstimulated cells (TF antigen 7 to 46 pg/10^6 cells, N_{TF}=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/10^6 cells, N_{TF}=5.94 to 23.43). Cultured monocytes did express FcγRII, 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 monocyctic 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;24:975-981.)

Key Words: C-reactive protein ■ tissue factor ■ monocytes ■ MCP-1 ■ atherosclerosis
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 Chemie 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 mM 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, St. Louis, Mo). Anti-CD14, anti-CD34, and anti-CD71 (Becton Dickinson). Total RNA was isolated using RNAeasy 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% Na-amidotrizoate, 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, 0.4 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 a dark atmosphere. Washed with binding buffer, and incubated with antihapten antibody-conjugated microbeads. The nonlabeled fraction containing the monocytes was recovered by use of a magnetic field.

#### 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 × 10⁶ 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, pelleted by centrifugation, and fixed with 100 μL 1% paraformaldehyde. Fluorescence was analyzed on a FACScan 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 fluorescent 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'-CCCCTGGCAAGTCGTGACTG-3') and sense and antisense primers specific for human TF mRNA (sense primer, 5'-GGAGCCCAACCCGTCAATTAC-3'; antisense primer, 5'-TCCGAGTTTGTCCTCCAGGT-3') or the TET(5')/TAMRA(3') fluorescent-labeled probe (5'-ACCAGCAGAAGTGGTCCTCGAAGCT-3') and sense and antisense primers specific for human MCP-1 mRNA (sense primer, 5'-GGTCAAGCCAGATGCAATC-3'; antisense primer, 5'-TTGTCCAGTGTCCTGATG-3'). β-Actin cDNA amplification was performed in the same way using (TET(5')/TAMRA(3') fluorescent-labeled probe 5'-TGATCGGTCGCTCTCAAGCCATCTC-3', sense primer, 5'-AGGCCACAGGGCTGTATAG-3'; and antisense primer, 5'-GCTTGAGATACGAAGCTATGCTTGG-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 35 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. The value for the number of cycles in which the fluorescence of the amplified fragment exceeds the fluorescent background threshold was designated by additional software (PE Applied Biosystems-Sequence Detection Systems 1.7). TF mRNA was expressed as the ratio of the number of TF transcripts relative to the number of β-actin transcripts (N<sub>TF</sub>/2<sup>ΔCt</sup>). 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 IA). 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 IA). Unstimulated cells did...
express similar levels of TF antigen (7 to 46 pg/10^6 cells, median of 12 pg/10^6 cells). Stimulation of Mo-FA with 10 μg/mL LPS resulted in strong induction of TF antigen (1125 to 6120 pg/10^6 cells, median of 3606 pg/10^6 cells) and TF cell surface expression (30% 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 (FcRγII 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, 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_{2}\text{Ct} 0.01 to 0.33, median NTF_{2}\text{Ct} 0.04) compared with unstimulated cells (NTF_{2}\text{Ct} 0.01 to 0.13, median NTF_{2}\text{Ct} 0.03). In contrast, addition of 10 μg/mL LPS resulted in a 300-fold increase in the level of TF mRNA (NTF_{2}\text{Ct} 5.94 to 23.43, median NTF_{2}\text{Ct} 9.29) compared with control cells (Table 1A). As expected, LPS-induced TF mRNA 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/106 cells, median 151 pg/106 cells) compared with unstimulated cells (24 to 53 pg/106 cells, median 25 pg/106 cells), but far less than expressed after stimulation with 10 μg/mL LPS (496 to 3151 pg/106 cells, median 1016 pg/106 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 amebocyte lysate assay, was able to elicit a significant response. 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 monocytes cells have a functional response system toward CRP. We tested this by measuring its effect on the secretion of monocyte 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, and also in human umbilical vein cells, CRP did induce MCP-1 expression. 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/106 cells with a median of 6.92), as well as increased MCP-1 mRNA levels (median N MCP-1 =1193) compared with unstimulated cells (0.12 to 7.62 ng/106 cells antigen with median 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/106 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. 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...
TABLE 2. Induction of TF Antigen and mRNA Expression in PBMCs by CRP and LPS

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<th>TF Ag</th>
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<td>Range (pg/10^6 cells)</td>
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<td>Control PBMC</td>
<td>0–27</td>
<td>4</td>
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<tr>
<td>CRP</td>
<td>41–543</td>
<td>154</td>
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<tr>
<td>LPS</td>
<td>499–1701</td>
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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 /H9004 Ct.

Discussion

It has been generally accepted that CRP induces TF expression in monocyte cells.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.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.6,7 In addition, CRP has been described to be a strong predictor for cardiovascular risk.10 Although many reviews refer to CRP-mediated induction of TF in monocytes cells,9,9 we were unable to demonstrate CRP-induced TF expression in highly purified human monocytes. We demonstrated that variations in CRP concentrations (20 to 100 μg/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 monocyte 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 μg 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.13,14 When CRP binds to phosphatidylcholine and LDL, it aids in opsonization and priming for phagocytosis.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.26,27 Indirect interaction of CRP by binding C1q present on the cell surface of monocytes 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.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.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-1α.42 MCP-1 is present in human atherosclerotic plaques.43 CRP-induced MCP-1 expression has been described before,43,44 but the mechanism of induced MCP-1 expression in human monocytes is not clear. Interestingly, MCP-1 has been reported to induce TF in monocytes45 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. 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. 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 IFNγ, secreted by activated T-lymphocytes, modulates TF expression of monocytes. Preliminary experiments did not reveal any effect of IFNγ 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-1β and TNFα. 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.

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References


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