Tissue Factor Is Induced by Monocyte Chemoattractant Protein-1 in Human Aortic Smooth Muscle and THP-1 Cells*

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Alison D. Schecter, Barrett J. Rollins, Yujun J. Zhang, Israel F. Charo, John T. Fallon, Maria Rossikhina, Peter L. A. Giesen, Yale Nemerson, and Mark B. Taubman

From the Cardiovascular Institute and Division of Thrombosis Research, Department of Medicine, the Department of Biochemistry, and the Division of Pathology, The Mount Sinai School of Medicine, New York, New York 10029, the Department of Medicine, the Dana Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, the Gladstone Institute of Cardiovascular Disease, San Francisco, California 94141, and the Cardiovascular Research Institute, Department of Medicine, University of California, San Francisco, California 94143

Monocyte chemoattractant protein-1 (MCP-1) is a C-C chemokine thought to play a major role in recruiting monocytes to the atherosclerotic plaque. Tissue factor (TF), the initiator of coagulation, is found in the atherosclerotic plaque, macrophages, and human aortic smooth muscle cells (SMC). The exposure of TF during plaque rupture likely induces acute thrombosis, leading to myocardial infarction and stroke. This report demonstrates that MCP-1 induces the accumulation of TF mRNA and protein in SMC and in THP-1 myelomonocytic leukemia cells. MCP-1 also induces TF activity on the surface of human SMC. The induction of TF by MCP-1 in SMC is inhibited by pertussis toxin, suggesting that the SMC MCP-1 receptor is coupled to a G-protein. Chelation of intracellular calcium and inhibition of protein kinase C block the induction of TF by MCP-1, suggesting that in SMC it is mediated by activation of phospholipase C. SMC bind MCP-1 with a Kd similar to that previously reported for macrophages. However, mRNA encoding the macrophage MCP-1 receptors, CCR2A and B, is not present in SMC, indicating that they possess a distinct MCP-1 receptor. These data suggest that in addition to being a chemoattractant, MCP-1 may have a procoagulant function and raise the possibility of an autocrine pathway in which MCP-1, secreted by SMC and macrophages, induces TF activity in these same cells.

Tissue factor (TF) is a transmembrane glycoprotein that

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& To whom correspondence should be addressed: Box 1269, Mt. Sinai School of Medicine, One Gustave L. Levy Place, New York, New York 10029. Tel.: 212-241-0047; Fax: 212-860-7032; E-mail: M_Taubman@mssm.edu.

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Pro-Arg-g-p-nitroaniline was synthesized in our laboratory.

**Cell Culture**—SMC were isolated from human thoracic aortas. The adventitia and connective tissue were removed; the remaining arterial intima and media were cut into 1-cm² segments and placed in culture dishes with 2.5 mg/ml collagenase and 15% FCS. Cells were grown in Dulbecco’s modified Eagle’s medium total Dulbecco’s medium containing 200 units/ml penicillin, and 100 μg/ml streptomycin. Cells were identified as smooth muscle by their typical appearance on light microscopy and by immunostaining with antibody to human smooth muscle α-actin (0.1 μg/ml) (Dako Corp., Carpinteria, CA). The lack of EC contamination of SMC was established by the absence of staining with antibody (0.03 μg/ml) to von Willebrand factor (Dako Corp., Carpinteria, CA). After treatment, SMC (passages 3–10) were determined to be free from SMC contamination by the absence of primary antibody were performed for each cell type. Man placenta) and negative controls using non-immune IgG or the mouse aortic SMC were isolated from human thoracic aortas. The slides were fixed in 4% paraformaldehyde (in phosphate-buffered saline), air dried, fixed, and mounted in a closed-circuit parallel-plate flow chamber and perfused continuously with 150 mM factor X and 1.0 nM factor VIIa in 10 mM sodium phosphate buffer pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA, and subsequently increasing concentrations of unlabeled M-199 were added to displace the 125I-labeled MCP-1. Measurements were performed in duplicate on two different preparations of human aortic SMC. For each experiment, the non-displaceable M* was estimated by fitting the free M⁺ versus M⁻ to Equation 1.

**Binding Studies**—Binding of MCP-1 to human SMC was performed as described (25). SMC (passage 3–9) were incubated with a fixed amount of 125I-labeled MCP-1 (M⁺) in binding buffer (50 mM HEPES, pH 7.2, 1 mM CaCl₂, 5 mM MgCl₂, 0.5% BSA), and subsequently increasing concentrations of unlabeled MCP-1 (M⁻) were added to displace the 125I-labeled MCP-1. Measurements were performed in duplicate on two different preparations of human aortic SMC. For each experiment, the non-displaceable M* was estimated by fitting the free M⁺ versus M⁻ to Equation 1.

**Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**—RT-PCR for all segments of the CCR2 gene was done with the following cycling conditions: for RT, RNA was incubated with antisense oligonucleotide at 70 °C for 10 min and then quick-chilled, and 200 units of Superscript II reverse transcriptase (Life Technologies, Inc., Gaithersburg, MD) were added with RT mix and incubated for 1 h at 50 °C and then another 40 min at 70 °C. All subsequent PCR reactions were performed under the following cycling conditions: initial incubation at 94 °C for 4 min, followed by 50 cycles of 45 s at 94 °C, 60 s at 55 °C, 75 s at 72 °C, and a final incubation for 10 min at 70 °C. RT-PCR products were ligated into the TA3 vector using the TA cloning kit (Invitrogen, San Diego, CA) and sequenced commercially (Biotechnology Center, Utah State University). One set of primer pairs, corresponding to nt 37–54 (sense) and 227–243 (antisense) of CCR2A, is common to the 5′-coding region of both receptors (22). For the second set, the antisense primer is unique to nt 1252–1274 of CCR2B. The sense primer (nt 363–380 of CCR2B) is common to both receptors. For the third set, the antisense primer is unique to nt 1246–1268 of CCR2A. The sense primer (nt 748–768 of CCR2A) is common to both. To investigate other CCR receptors, the following primers were synthesized: CCR1 (26) (sense, nt 1210–1229; antisense, nt 1404–1387), CCR3 (27) (sense, nt 1102–1119; antisense, 1201–1218), CCR4 (28) (sense, nt 1390–1408; antisense, 1201–1229; antisense, nt 1404–1387), and CCR5 (29) (sense, nt 1985–2004; antisense, 2243–2262). As a control for the quality of the human SMC RNA, RT-PCR was also performed with primers spanning nt 320 and 760 of the human TF cDNA (24).

**Statistics**—Experiments were performed at least 3 times and values presented as the mean ± S.E. Student’s t test was used in the analysis of paired and unpaired means. Unless indicated, all values were significant at p < 0.05.

**RESULTS**

**Induction of TF mRNA and Antibody by MCP-1—Quiescent human aortic SMC contained low levels of TF mRNA. Exposure**
Tissue Factor Is Induced by MCP-1 in Human SMC

of these SMC to MCP-1 resulted in the accumulation of TF mRNA, peaking at 4 h and returning to base line by 8 h (Fig. 1A). The levels of TF mRNA were comparable to those induced by serum. The accumulation of TF mRNA by MCP-1 was concentration-dependent and was seen with as little as 0.1 ng/ml MCP-1 (Fig. 1B). Unstimulated THP-1 cells contained low levels of TF mRNA. Exposure of THP-1 cells to MCP-1 resulted in accumulation of TF mRNA levels comparable to the induction of TF seen with lipopolysaccharide (LPS) (Fig. 1C).

By immunohistochemical analysis, quiescent SMC contained minimal amounts of TF antigen (Fig. 2A). Exposure to as little as 0.01 ng/ml MCP-1 resulted in the accumulation of TF antigen around the nucleus at 4 h (Fig. 2B). Significantly, accumulation of TF antigen was seen in virtually all cells, suggesting that the response to MCP-1 was uniform and not limited to a subpopulation of SMC or a small number of contaminating fibroblasts. Accumulation of perinuclear TF at 4 h was also seen when SMC maintained in 10% FCS were treated with MCP-1. Concomitant treatment with MCP-1 and the transcription inhibitor actinomycin D markedly diminished the perinuclear accumulation of TF antigen (Fig. 2C). Addition of actinomycin D 1 h after MCP-1 treatment had no effect on the perinuclear accumulation of TF antigen. In further experiments, SMC were treated with MCP-1 and at varying times the

medium was removed, the cells washed with phosphate-buffered saline, and fresh serum-free medium added. TF antigen was examined 4 h after the initial exposure. Only 30 min of exposure to MCP-1 was required for the accumulation of TF antigen around the nucleus (not shown).

MCP-1 also induced TF mRNA (Fig. 1C) and antigen (Fig. 2, D–F) in human THP-1 myelomonocytic leukemia cells to an extent similar to that of LPS (not shown) and phorbol 12-myristate 13-acetate (PMA), known activators of TF in these cells (30). To rule out the possibility that the induction of TF by MCP-1 was due to contaminating LPS, SMC and THP-1 cells were treated with 20 mg/ml polymyxin B. Polymyxin, which inhibits many of the actions of LPS, failed to inhibit the induction of TF by MCP-1 (31). MCP-1 did not induce TF antigen in EC (Fig. 2, G–I). This suggests that the response to MCP-1 is cell type-restricted and further argues against contamination with LPS, a potent inducer of TF in EC (32).

**Induction of TF Activity by MCP-1**—To determine whether MCP-1 induced active TF on the cell surface, TF activity (factor Xa generation) was measured from SMC monolayers perfused with purified clotting factors in a parallel plate chamber. Four hours after treatment with MCP-1 (1 ng/ml), surface TF activity was 2.6 ± 0.8 × that seen with DM alone (p < 0.05; n = 6). This corresponds to ~0.2 fmol/cm² of surface TF generated in response to MCP-1 and is similar to that seen with 10 ng/ml PDGF AB.²

MCP-1 also induced a 29 ± 9% increase in total cellular TF activity at 4 h. (n = 10). This was comparable to that found with treatment with 10 ng/ml PDGF AB (49 ± 19%; n = 6).

**Analysis of the CCR2 Receptors in Human SMC**—Two MCP-1 receptors, generated by alternative splicing of a single gene, have been cloned from human monocytes (22, 25). The presence of functional MCP-1 receptors has been described solely on cells of myelomonocytic origin. The above results suggest that human arterial SMC also possess a functional MCP-1 receptor. Accordingly, specific binding of MCP-1 to human aortic SMC was examined (Fig. 3). The Kᵰ, which was derived from a specific binding curve, was approximately 1.0 nM, comparable to the affinity of MCP-1 for human macro-

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² Schecter, A. D., Giesen, P. L. A., Taby, O., Rosenfield, C. L., Rossikhina, M., Fyfe, B. S., Khotz, S., Fallon, J. T., Nemerson, Y., and Taubman, M. B. (1997) *J. Clin. Invest.* in press.
To determine whether the MCP-1 receptors (CCR2A and B) cloned from macrophages were expressed in human SMC, RNA blot analysis was performed using a 2.2-kb fragment of the human CCR2B cDNA that hybridizes to both alternatively spliced forms (Fig. 4A). SMC RNA (S) hybridized to a band of higher molecular weight than the CCR2 mRNA found in THP-1 cells (T); this higher molecular weight band hybridized only at very low stringency. No band was seen by RT-PCR of SMC RNA using primers derived from either the shared 5' end of CCR2A and B mRNAs or specific to the 3' end of CCR2A (Fig. 4B, lanes 1 and 5, respectively). Primers specific to the 3' end of CCR2B yielded a band of lower molecular weight than the expected product (lane 3). The sequence of this 600-bp band was not present in the GenBank and had no significant homology to CCR2 or other C-C chemokine or G-protein-coupled receptors. In addition, the 600-bp RT-PCR product hybridized predominantly to an ~1.2-kb mRNA (not shown) and not to the ~5-kb species detected in SMC in Fig. 4A. These data suggest that the 600-bp band is an artifact of RT-PCR and does not represent the SMC MCP-1 receptor. To investigate whether human aortic SMC possess the other known C-C chemokine receptor, RT-PCR was also performed with primers specific to CCR1, CCR3, CCR4, and DARC. No signals were seen with any of these primers (not shown).

Analysis of Signaling Pathways Involved in the Induction of TF by MCP-1—CCR2A and B belong to the family of seven transmembrane spanning, pertussis-sensitive heterotrimeric G-protein-coupled receptors that mobilize intracellular calcium (Ca^{2+}) (34). Treatment of quiescent SMC with pertussis toxin prior to MCP-1 exposure blocked the accumulation of TF mRNA in response to MCP-1 or PDGF. In contrast, the addition of BAPTA/AM to chelate Ca^{2+} completely blocked the accumulation of TF mRNA (Fig. 5) and protein (Fig. 6B), suggesting that Ca^{2+} mobilization is involved in the induction of TF by MCP-1. Previous studies have suggested that the induction of TF by PDGF in rat SMC (35) does not depend on the activation of protein kinase C (PKC). SMC were treated for 24 h with PDBu to down-regulate cytoplasmic PKC activity (36). This blocked the accumulation of TF mRNA in response to MCP-1 but not to PDGF BB (Fig. 5B). As expected, PDBu pretreatment blocked the accumulation of TF mRNA in response to PMA, a direct activator of PKC (Fig. 5B). Staurosporine, a nonspecific inhibitor of PKC, also markedly reduced TF antigen accumulation in response to MCP-1 (Fig. 6C).

It should be noted that in some experiments, a 3.2-kb mRNA species was seen in addition to the ~2-kb TF mRNA. This larger mRNA has been shown to be produced by the retention of the first intron of the human TF gene and does not encode a functional TF protein (1). The higher molecular weight species was detectable largely when levels of the 2.2-kb mRNA were high and its intensity (as determined by densitometry) varied from 10 to 100% of the 2.2-kb mRNA. The 3.2-kb mRNA was most prominent in cells treated with phorbol ester or EDTA. Although the mobilization of Ca^{2+} may not be necessary for transcription of TF mRNA, it may be important for correct regulation of mRNA processing and splicing.
DISCUSSION

This report describes the induction of TF mRNA, antigen, and activity in human aortic SMC by the C-C chemokine, MCP-1. This is the first report, to our knowledge, demonstrating that MCP-1 is an agonist for non-leukocytes and, in particular, that human arterial SMC possess a functionally coupled MCP-1 receptor.

Two MCP-1 receptors, generated by alternative splicing and designated as CCR2A and -B, have been cloned in human monocytes (22). On the basis of PCR studies, the SMC MCP-1 receptor is distinct from these two receptors. In addition, it is unlikely that the SMC MCP-1 receptor is generated by alternative splicing of the same gene, because no signal was seen on RNA blot analyses at moderate stringency washings using a random-primed probe encompassing the entire coding region. The SMC MCP-1 receptor would thus need to be spliced very differently from that of the CCR2A and -B isoforms; more likely it is derived from a different gene, but this remains to be demonstrated. Additional PCR studies employing primers from the other cloned human C-C chemokine receptors also failed to produce a signal. Because these studies were not exhaustive, they do not rule out the possibility that the SMC contain an alternatively spliced form of another known C-C chemokine receptor. It should be noted that these other cloned receptors are not known to bind MCP-1 with high affinity. It is therefore likely that the SMC MCP-1 receptor is different from previously cloned C-C chemokine receptors. Whether the ~5-kb mRNA identified by hybridization at low stringency with the CCR2 cDNA (Fig. 4A) encodes the SMC MCP-1 receptor remains to be determined.

The two alternatively spliced MCP-1 receptors, CCR2A and -B, belong to the family of heptahelical, pertussis-sensitive G-protein-coupled receptors (33). The SMC MCP-1 receptor also appears to be coupled to G_{i,3} protein activation because the induction of TF was inhibited by pertussis toxin. A common pathway in receptor-mediated signal transduction in SMC is the cleavage of plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_{2}) by phospholipase C to produce inositol-1,4,5-trisphosphate (IP_{3}) and 1,2-diacylglycerol (37). IP_{3} mobilizes Ca^{2+} (38), whereas 1,2-diacylglycerol activates PKC (39). MCP-1 has been shown to mobilize Ca^{2+} in a number of studies (40–42). However, the role of phospholipase C in mediating CCR2A and -B signal transduction has been questioned. In cells stably transfected with CCR2B (33) and in peripheral monocytes (43), MCP-1 treatment failed to cause hydrolysis of PIP_{2}. It was postulated that the CCR2 receptors either mobilized Ca^{2+}, via an IP_{3}- and phospholipase C-independent mechanism (33) or that PIP_{2} hydrolysis occurred at undetectable levels (44).

Studies investigating the role of PKC activation in CCR2-mediated signal transduction also have been mixed. One study (45) demonstrated significant inhibition of monocyte chemotaxis by the PKC inhibitors, staurosporine and H-7. However, treatment of human monocytes with PMA, a direct PKC activator, failed to increase cytosolic Ca^{2+} (43) in response to MCP-1. Furthermore, pretreatment with staurosporine or H-7 failed to inhibit MCP-1-induced Ca^{2+} influx (43). In the present study, the induction of TF by MCP-1 in human SMC required Ca^{2+} mobilization and was PKC-dependent, suggesting a role for phospholipase C activation in the induction of TF. It remains to be determined whether the SMC MCP-1 receptor signals via an IP_{3}-dependent pathway or mobilizes Ca^{2+} in an IP_{3}-independent fashion as proposed for CCR2A and -B.

The present study also demonstrates a divergence in PDGF and MCP-1 signaling in that the induction of TF by MCP-1 was inhibited by down-regulation of PKC by phorbol esters, whereas the induction by PDGF was not. The PKC family of enzymes consists of a complex group of highly related isoforms that can be distinguished by Ca^{2+} dependence, differential activation of diacylglycerol, and phorbol ester responsiveness (46). Only α, β, ε, δ, and ζ isoenzymes have been identified in smooth muscle (37, 47). Previous studies have suggested that PDGF signals via the atypical isoform ζ in SMC (48) and in other cell types (49). PKC ζ is the likely mediator for the PDGF induction of TF, given that this isoform is not down-regulated by prolonged pretreatment with phorbol (48). MCP-1 signaling is Ca^{2+}-dependent and therefore is more likely to be mediated by the classical PKC subgroups α, β, or γ.

TF is induced by growth factors and cytokines as part of the “immediate-early” gene program in a variety of cells, including 3T3 fibroblasts, rat aortic SMC, THP-1 cells, human monocytes, and human endothelial cells (reviewed in Ref. 1). The regulation of the human TF promoter has been shown to involve a number of different elements, working either cooperatively or singly, depending upon the agonist used. In COS-7 and HeLa cells, the minimal TF promoter spans a 111-bp region that contains a serum response region requiring the cooperative action of three SP-1 sites (50, 51). In contrast to serum, the response to lipopolysaccharide in THP-1 and endothelial cells requires the cooperative activity of two AP-1 sites and an NFκB site (52, 53). The transcription factors responsible for inducing TF in these cell types are thus ubiquitous and involved in the induction of other immediate-early genes. Similarly, the signaling pathways involved in the induction of TF by MCP-1 are employed by many other agonists involved in gene induction. Although TF was examined in this study as a target for MCP-1-mediated responses in SMC, our initial signaling studies and previous analyses of the TF promoter suggest that MCP-1 is likely to induce other genes in SMC and may activate much of the immediate-early gene program.

In the present study, TF activity (Xα generation) was measured on the surface of unperturbed SMC monolayers under flow conditions at low shear rate (54, 55). Studies (56–61) have suggested that for some cell types including SMC (56), much of the TF activity exists in a latent or “encrypted” form. By disrupting the cell membrane or by dissociating cells from their matrix, the latent TF becomes accessible. This augmentation of TF activity with cell disruption has been postulated to be due to changes in the orientation and structure of anionic phospholipid in the cell membrane (58). The presence of TF in caveolae (56, 59), which undergo conformational changes and expose...
more TF when cells are placed in suspension, is another proposed mechanism for release of encrypted TF. Our approach minimizes cellular disruption and maintains SMC in a monolayer together with the extracellular matrix. It also allows for the measurement of TF activity under conditions that are not diffusion rate-limited (54). These results, therefore, may more accurately reflect the TF accessible on the cell surface under physiologic conditions. Under these circumstances, MCP-1 caused an ∼3-fold increase in surface TF activity at 4 h.

The effect of MCP-1 and other agonists on total cellular TF activity is less pronounced than their effects on surface activity. This may be explained in part by the high level of “baseline” TF activity found in cultured human SMC lysates. This base line is due to the presence of encrypted TF on the cell surface and to intracellular stores of TF.2 It is therefore difficult to measure a significant induction in total TF activity upon growth factor stimulation. However, small changes in total cellular TF activity are accompanied by substantial increases in the expression of active TF on the cell surface.

MCP-1 protein has been identified in early human atherosclerotic lesions (62) and in advanced human, primate, and rabbit atherosclerotic plaques (14, 15, 63). In the primate model, the medial and intimal SMC contain high levels of MCP-1 mRNA and protein (63). In human plaques, MCP-1 mRNA is localized in macrophage-rich regions (14) and around SMC (64). In human atherosclerotic plaques, TF has been found in the lipid-rich core, in macrophages and in SMC (3–5), areas which are rich in MCP-1 protein. MCP-1 (21) and TF (4) are rapidly induced in the arterial media and accumulate in the developing neointima after experimental balloon injury. MCP-1 has been thought to play an important role in recruiting macrophages to the atherosclerotic plaque. This study suggests that in addition to this role, MCP-1 also may induce procoagulant activity. These findings raise the possibility of an auto-irritant pathway in which MCP-1, secreted by SMC and macrophages, induces TF in the same cells. This pathway may provide an important target for inhibiting intra-arterial and plaque thrombosis.

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