Monocyte chemoattractant protein-1 (MCP-1) belongs to the β or C-C subfamily of chemotactic cytokines, also known as the chemokines (or intercrines). The expression of this 76-amino acid monomeric protein has been detected in various human diseases, including atherosclerosis, skin delayed-type hypersensitivity reactions, cancer, rheumatoid arthritis, and renal failure. MCP-1 has largely been associated with modulating monocyte migration in response to inflammation. MCP-1 has been shown to induce Ca\textsuperscript{2+} influx, integrin and tissue factor expression, arachidonic acid release, and cytokine expression in monocytes. MCP-1 has also been shown to stimulate basophils to release histamine and a subset of lymphocytes to migrate. Recently, additional functions of MCP-1 have been revealed, including blockage of M-tropic virus entry into blood dendritic cells and protection of mice from lethal endotoxemia. In this regard, MCP-1 induction may be of clinical relevance.

MCP-1 expression is induced in a wide variety of cells in vitro, including monocytes, fibroblasts, and vascular endothelial and smooth muscle cells, in response to various extracellular stimuli such as tumor necrosis factor-α, leukemia inhibitory factor, IL-1, IL-4, IL-6, IL-15, lipopolysaccharide (LPS), platelet-derived growth factor, or interferon-γ. In contrast to extracellular ligands, Burn et al\textsuperscript{13} has shown that all-trans retinoic acid (ATRA), a ligand for retinoic acid receptor (RAR), also elevates expression of MCP-1 in human HL-60 and NB4 cells. RA is a member of the nuclear hormone receptor superfamily. On ligand activation, RAR modulates the expression of its target genes by binding to specific DNA elements in their promoter regions.\textsuperscript{35} ATRA also promotes the differentiation of HL-60 and NB4 cells to neutrophil-like cells. Thus, whether increased MCP-1 expression is a direct effect of ATRA or a consequence of differentiation in those studies is unknown. In this regard, these investigators have also shown that MCP-1 expressed in human peripheral blood neutrophils is not further induced by ATRA.\textsuperscript{35}

To examine the regulation of MCP-1 expression in undifferentiated human monocytes, human THP-1 cells, which highly express retinoid X receptor α (RXRα)\textsuperscript{36} were cultured with 9-cis retinoic acid (RA). RA activates signaling pathways through RXR-RXR homodimers, and both liver X receptor-RXR (LXR-RXR)\textsuperscript{39} and PPAR-RXR heterodimers.\textsuperscript{40–42} RA has also been shown to induce PPARγ expression in undifferentiated THP-1 cells.\textsuperscript{43} It has recently been reported that PPARγ plays an important regulatory role.
in monocyte and macrophage inflammatory actions, as well as in lipid-laden foam cell transformation. In the current study, RA was used to examine whether the retinoic acid signaling pathway regulates MCP-1 expression in THP-1 cells as well as in monocytes isolated from human blood. Our data indicates RA may act as a nuclear signal for induction of MCP-1 expression.

Methods

Chemicals

RA was purchased from Sigma Chemical Co. Wy 14,643 was purchased from Chemsyn Science Laboratory. 15d-PGJ₂ was purchased from Cayman Chemical Company. BRL49653, troglitazone, and PD195599 (4-(3-(2-propyl-3-hydroxy-4-acetyl-phenoxy)propyloxy)phenoxy acetic acid, Merck compound A) were synthesized at Parke-Davis Pharmaceutical Research, Ann Arbor, MI.

Cell Culture

Human THP-1 cells were obtained from the American Type Culture Collection, Manassas, VA. Cells were cultured in RPMI 1640 medium (Gibco BRL) containing 10% fetal bovine serum and 0.05 mmol/L 2-mercaptoethanol (Gibco BRL). For RA and PPAR ligands treatments, cells (1x10⁵ cells/mL) were switched to differentiation medium (DM) containing 1% Nutridoma-Hu (Boehringer Mannheim) and 0.05 mmol/L 2-mercaptoethanol in RPMI 1640 medium containing the ligands in dimethylsulphoxide (DMSO; 0.2% of final volume). Frozen elutriated human monocytes (Advanced Biotechnologies Inc, Md) were thawed, plated at 1x10⁵ cells/mL and cultured for 1 day in RPMI 1640 medium (Gibco BRL) containing 20% fetal bovine serum. DMSO, RA, or BRL49653 were then directly added to the culture medium.

Migration Assay

THP-1 cell migration assay was performed using 6.5-mm Costar Transwell cell culture chamber with polycarbonate membrane (5.0 micron pore) following the manufacturer’s protocol. MCP-1 protein (R&D Systems, Minneapolis, Minn) was dissolved in RPMI1640 medium with 0.2% BSA and placed in lower compartment. THP-1/F4-2 cell suspensions of 7x10⁵ cells/mL in RPMI1640 medium with 0.2% BSA were first treated with either DMSO vehicle control or 500 nmol/L RA for 12 hours and then loaded in the upper
compartment. After 3-hour incubation at 37°C in a humidified atmosphere with 5% CO₂, the number of cells migrated to the lower compartment was counted with a Coulter Z1 particle counter.

**RNase Protection Assays**
Total cellular RNA was isolated from THP-1 cells treated with RA for 24 hours using TRIzol reagents (Gibco BRL). A 32P-labeled antisense riboprobe for human MCP-1 was prepared using a RiboQuant In vitro Transcription Kit (PharMingen) with the hCK-5 RiboQuant Human Cytokine Multi-Probe Template Set (PharMingen), which contains the DNA template for human MCP-1 cDNA. A 32P-labeled antisense riboprobe for human MCP-1 receptor (CCR-2) was prepared using the RiboQuant In vitro Transcription Kit with the hCR-5 RiboQuant Human Cytokine Multi-Probe Template Set (PharMingen) which contains the DNA templates for human CCR-2a and CCR-2b cDNA. RNase protection assays were performed using an Ambion RPA II RNase protection assay kit (Ambion). RNase protection results were quantified by NIH Image version 1.61.

**MCP-1 ELISA**
The concentration of MCP-1 released into the medium was determined by ELISA of culture supernatants. MCP-1 protein was measured with a Quantikine Human MCP-1 Immunoassay ELISA kit (R&D Systems) following the manufacturer’s protocol. Data were analyzed using Softmax PRO software (Molecular Devices).

**Biotinylated MCP-1 Binding Assay**
MCP-1 binding assays were performed using FLUOROKINE Human MCP-1 Biotin Conjugate Flow Cytometry Reagents (R&D Systems) according to the manufacturer’s protocol. Briefly, treated THP-1 cells were washed twice with Dulbecco’s PBS (w/o Ca²⁺, Mg²⁺) and then stained with biotinylated Human MCP-1. Biotinylated soybean trypsin inhibitor, supplied with the kit, was used as a negative control. Cells were then treated with avidin-fluorescein and analyzed by flow cytometry on a FACScan (Becton Dickinson). Data were analyzed with CellQuest software (Becton Dickinson).

**Statistical Methods**
All data were expressed as mean ± SD. Statistical significance was determined with Tukey’s multiple comparison procedure applied after a one-way ANOVA conducted on data. The level of statistical significance for all tests was P<0.05.

**Results**
Regulation of MCP-1 expression by the retinoic acid signaling pathway was examined in human monocytic THP-1 cells cultured with RA. Cells treated with RA (0.05 to 500 nmol/L) for 24 hours released MCP-1 into the medium in a concentration-dependent manner reaching a 165-fold increase at the highest concentration of RA tested (Figure 1A). MCP-1 induction was rapid, indicated by a substantial increase of MCP-1 concentration in 500 nmol/L RA-treated cells (Figure 1B). MCP-1 RNA was also induced in the RA-treated THP-1 cells. After 24 hours, RA treatment caused a concentration-dependent and dramatic induction of MCP-1 RNA (6.7-fold induction in 5 nmol/L RA-treated cells and 12.4-fold induction in 500 nmol/L RA-treated cells) as revealed by a RNase protection assay (Figure 2). Under these conditions, THP-1 cells do not differentiate into macrophages.

To determine whether MCP-1 induction was dependent on ligand interaction with PPARγ, THP-1 cells were exposed to DMSO vehicle, or 1 or 10 μmol/L BRL49653 (a ligand for PPARγ) in the presence or absence of 500 nmol/L RA (Figure 3). BRL49653 failed to induce MCP-1 secretion alone and did not significantly alter the response observed after RA treatment. Although reduced MCP-1 secretion was observed at 10 μmol/L BRL49653, significance was not achieved. Additional PPAR ligands were also tested to determine whether they could modulate RA induction of MCP-1. THP-1 cells were cultured with 15d-PGJ2 (3 μmol/L), troglitazone (15 μmol/L), Wy14,643 (100 μmol/L), or PD195599 (20 μmol/L) alone (A) or in combination with 500 nmol/L RA (B). MCP-1 released into medium was determined by ELISA after cells were cultured for 24 hours with indicated agents. Data shown for both A and B are average ± SD from 3 independent experiments each performed in triplicate. * indicates significant difference from DMSO control treatments.

Figure 4. PPAR ligands, 15d-PGJ2 and troglitazone (PPARγ), Wy14,643 (PPARα), or PD195599 (PPARβ) inhibit RA’s effect on MCP-1 induction. THP-1 cells (1x10⁶ cells/mL) were cultured with 15d-PGJ2 (3 μmol/L), troglitazone (15 μmol/L), Wy14,643 (100 μmol/L), or PD195599 (20 μmol/L) alone (A) or in combination with 500 nmol/L RA (B). MCP-1 released into medium was determined by ELISA after cells were cultured for 24 hours with indicated agents. Data shown for both A and B are average ± SD from 3 independent experiments each performed in triplicate. * indicates significant difference from DMSO control treatments.
BRL49653 (1 or 10 μmol/L) were essentially the same as with BRL49653 alone (data not shown). In RA-treated THP-1 cells, a local increase in extracellular MCP-1 may act in an autocrine manner and cause receptor internalization. An apparent lack of an effect of RA on MCP-1 cell surface receptor expression may be due to this antocrine regulation. Indeed, MCP-1 receptor RNA was actually increased in the RA-treated THP-1 cells (Figure 6).

Human elutriated monocytes were also assessed for their ability to produce MCP-1 in response to RA. Cells were treated with DMSO, 500 nmol/L RA, or 10 μmol/L BRL49653 for 12 hours. MCP-1 secretion was induced (~15-fold) in the RA-treated cells (Figure 7).

To determine whether RA induction of MCP-1 impeded the chemotactic response, cell migration assays were performed using CCR-2 (the MCP-1 receptor) preselected...
THP-1 cells. Without preselection, we observed no effect of MCP-1 on chemotaxis (data not shown). Similarly, Vaddi et al reported the inability to demonstrate chemotaxis in unselected THP-1 cells. Therefore, cells were selected for CCR-2 for migration assays (THP-1/F4-2 subpopulation). In the absence of exogenous MCP-1, 500 nmol/L RA pretreatment (12 hours) resulted in a 600% inhibition in the ability of the THP-1/F4-2 to migrate to the lower chamber. Exogenous MCP-1 in the lower chamber resulted in enhanced migration of the DMSO- or RA-treated THP-1/F4-2 cells. However, as the concentration of MCP-1 increased in the lower chamber, the inhibitory effect of RA on migration was mitigated (Figure 8A). The expression of MCP-1 receptor on the THP-1/F4-2 cell surface was also not affected by the RA treatment (Figure 8B).

Discussion

MCP-1 plays a critical role in the regulation of human monocyte function; however, the mechanism by which MCP-1 expression is regulated is not fully understood. Although various ligands known to bind cell surface receptors have been shown to induce MCP-1 expression, the nuclear events responsible for increased expression are poorly understood. In the current study, we discovered that RA, a ligand for the nuclear hormone receptor RXR, produces a massive induction of MCP-1 RNA as well as the secreted protein from THP-1 cells. RA also induces PPARγ expression in these cells, and this receptor can heterodimerize with RXR. RXR can also heterodimerize with LXR and form homodimers. Recently, it was reported that PPARγ ligands regulate monocyte and macrophage in inflammatory actions.

In the current study, addition of a ligand to PPARγ (BRL 49653) did not alter the MCP-1 expression level observed with RA alone (Figure 3). Other PPAR ligands, including 15d-PGJ2 and troglitazone (PPARγ ligands), Wy14,643 (PPARα ligand), and PD195599 (PPARβ ligand) inhibited the RA’s induction of MCP-1 secretion (Figure 4B). These data suggest that the RA induction of MCP-1 expression is not mediated through PPAR activation. Although MCP-1 expression is similarly induced by PMA, LPS, or RA, MCP-1 binding to the cell surface differs. Both PMA and LPS suppress, whereas RA has no effect on binding of MCP-1 to monocytes. The THP-1 cells treated with RA secrete MCP-1 and also express the MCP-1 receptor. Therefore, RA treatment may result in an autocrine regulation. Indeed, RNA from RA-treated cells show an increase in MCP-1 receptor RNA despite no detectable increase in MCP-1 cell surface receptors. Interestingly, BRL49653 alone significantly increased the MCP-1 binding to THP-1 cells, although BRL49653 failed to alter MCP-1 expression in this same system. Overall, the data presented demonstrate that MCP-1 function may be differentially regulated by PPAR and RA signaling pathways.

The findings of the current study provide evidence that RA-RXR may be the intracellular/nuclear signaling pathway for MCP-1 induction. Whether extracellular signals that also increase MCP-1 expression act via ligand activation of RXR is currently not known. Inhibition of MCP-1 induction in experiments with RXR-RA interactions are blocked, by either antagonism or by the creation of a RXR deficiency, may yield direct evidence for the existence of the nuclear pathway.

Markedly enhanced monocyte MCP-1 expression may have functional relevance. High secretion of MCP-1 could create a localized increased cytokine concentration, thereby making the monocyte resilient to concentration gradients that
therapeutic utility in the treatment of atherosclerosis diseases. Nuclear ligands that regulate MCP-1 expression may have positive effects on monocytes to migrate. Our finding that blocking MCP-1 could completely override the suppressive migration effect RA had on THP-1 cells. Perhaps RA modulates an antichemotactic response, decreasing the infiltration of monocytes to an inflammatory site. Our finding that blocking of MCP-1 could completely override the suppressive migration effect RA had on THP-1 cells. Perhaps RA modulates an antichemotactic response, decreasing the infiltration of monocytes to an inflammatory site.

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References


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Lingyu Zhu, Charles L. Bisgaier, Michael Aviram and Roger S. Newton

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