Monocyte Chemoattractant Protein-1 Gene Is Expressed in Activated Neutrophils and Retinoic Acid-Induced Human Myeloid Cell Lines

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We have used differential display polymerase chain reaction to identify genes that are upregulated after retinoic acid (RA) treatment of human myeloblastic HL-60 cells. Three of the cDNAs cloned hybridized to RA-inducible transcripts on Northern blots, one of which was shown to encode sequences for monocyte chemoattractant protein-1 (MCP-1), a recently described cytokine that is chemotactic for monocytes but not for neutrophils. Nuclear run-on analysis indicated that the upregulation of the MCP-1 gene occurs at the transcriptional level in HL-60 cells. MCP-1 transcript levels also increased after RA treatment of the NB4 acute promyelocytic cell line. MCP-1 transcripts were undetectable in freshly isolated neutrophils by Northern analysis or reverse transcription-polymerase chain reaction but were readily detectable in neutrophils after incubation in media at 37°C for 20 hours, suggesting that an activation event can lead to MCP-1 expression in neutrophils. Immunocytochemistry confirmed the presence of MCP-1 protein in activated neutrophils. This is the first report that the MCP-1 gene is RA-responsive in myeloid cell lines and is expressed in neutrophils. MCP-1 expression by activated neutrophils may play an important role in attracting monocytes to the site of tissue damage or infection.

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MATERIALS AND METHODS

Cell culture. HL-60 (CCL-240; American Type Culture Collection, Rockville, MD) were maintained in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS; testes endotoxin free: HyClone, Logan, UT) and 2 mmol/L L-glutamine. NB4 cells (obtained from M. Lanotte, INSERM, Hôpital Saint-Louis, Paris, France) were grown in RPMI and 10% FCS. HL-60 cells were induced with 10−6 mol/L ATRA (Sigma Chemical Co, St Louis, MO). NB4 cells were induced with 10−7 mol/L ATRA.

DD-PCR. DD-PCR was performed essentially as described.16,20 Total RNA was isolated from RA-induced HL-60 cells using the guanidinium isothiocyanate-CsCl method.29 DNAseI digestion was performed on 50 μg of total RNA at 37°C for 30 minutes in a 55-μL reaction volume containing 1 × PCR buffer (10 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KC1, 1.5 mmol/L MgCl2, 0.001% gelatin), 40 U RNasin (Promega, Madison WI), 20 U DNAseI (GIBCO-BRL, Gaithersburg, MD). The reaction was phenol/chloroform/isoamyl alcohol (24:24:1)-extracted, and ethanol was precipitated. Reverse transcription (RT) was performed at 37°C for 60 minutes using 200 ng of DNAseI-treated RNA in a 20-μL reaction containing 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (GIBCO BRL), 1 μmol/L of an anchored T₇mer containing two

A growing body of evidence suggests that retinoic acid (RA) plays a significant role in human myeloid differentiation through its activation of RA receptor-α (RAR-α), which is the predominant RAR in hematopoietic tissues.1 RA has been shown to inhibit proliferation and to induce terminal neutrophilic differentiation of the human myeloblastic cell line HL-60.2 RA can also stimulate granulocyte production while inhibiting monocyte and erythroid differentiation in vitro differentiation assays using purified CD34+ progenitors.3,4 Expression of a dominant negative RAR-α can block RA-induced neutrophil differentiation of HL-60 cells5 as well as differentiation of a multipotential cell line at the promyelocytic stage of differentiation.6 In addition, all-trans RA (ATRA) can induce leukemic cells from patients with acute promyelocytic leukemia to differentiate into mature neutrophils both in vivo and in vitro.8,10 At the molecular level, acute promyelocytic leukemia is characterized by a chromosomal translocation between the PML gene on chromosome 15 and the RAR-α gene.1 However, despite a significant body of evidence implicating RA in hematopoi-
non-T residues at the 3’ end of the oligo (as described),12,20 10 mmol/L dithionebitol (DTT), 20 µmol/L dNTPs (Pharmacia, Piscataway, NJ) and 1× first strand synthesis buffer (50 mmol/L Tris-HCl pH 8.3), 75 mmol/L KCl, 6 mmol/L MgCl2. The reaction was terminated by incubation at 95°C for 5 minutes.

Forty cycles of PCR (94°C for 40 seconds, 40°C for 2 minutes, and 72°C for 30 seconds, followed by a 5-minute elongation at 72°C) were performed in a 20-µL reaction containing 1× PCR buffer, 2 µL of RT product, 2 µmol/L dNTPs, 1 µmol/L of the same T1 primer used in the RT, 1× µmol of 5’ primer (10mer selected at random as described)12,13, 5 µCi of 1200 Ci/mmol (α-32P) deoxyadenosine triphosphate (dATP; Dupont NEN, Boston, MA), and 1 U of Taq polymerase (Perkin Elmer-Cetus, Norwalk, CT). A total of 4 µL of stop mix (95% formamide, 20 mmol/L EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added to 3.5 µL of the PCR reaction, the mix was heated at 80°C for 3 minutes, and the products were separated on a 6% denaturing sequencing gel.

Bands that were differentially expressed in two independent DD-PCR reactions were excised from the dried gel and placed in an Eppendorf tube containing 100 µL of autoclaved H2O. After a 10-minute room temperature incubation to hydrate the gel, the suspension was boiled for 10 minutes. Gel fragments were removed by centrifugation for 5 minutes, and the DNA was recovered by ethanol precipitation using 50 µg of glycerol (Boehringer Mannheim Biochemicals, Indianapolis, IN) as a carrier. The resulting pellet was resuspended in 10 µL of autoclaved H2O. Secondary PCR was performed in a 50 µL reaction containing 1× PCR buffer, 5 µL of eluted DNA, 20 µmol/L dNTPs, and 1 µmol/L of each of T1mer and 10mer. Reamplified PCR products were subcloned into the pGEM-T vector (Promega) as recommended by the manufacturer. DNA sequence was determined by using the dideoxy-chain termination method.23

Northern analysis. Total RNA was isolated by the guanidinium isothiocyanate/CsCl method24 and separated on 0.22 mol/L formaldehyde/1% agarose gels. Equal loading was verified by ethidium bromide staining of the 28s and 18s ribosomal RNA bands. RNA was transferred to Biortran membrane (ICN, Costa Mesa, CA) by capillary blotting in 20× SSC (1× is 0.15 mol/L NaCl, 0.015 mol/L sodium citrate). Membranes were baked for 1 hour at 80°C in a vacuum oven and were UV–cross-linked for 5 minutes. DD-PCR products were labeled to specific activity of at least 108 dpm/µg using the random hexamer method.25 Prehybridization and hybridizations were performed in 0.5 mol/L NaPO4 (pH 7.2), 7% sodium dodecyl sulfate (SDS), and 1% bovine serum albumin at 65°C. Membranes were washed in 0.2× SSC/0.1% SDS for 5 minutes at room temperature followed by 30 minutes at 65°C. Exposures were at ~80°C with an intensifying screen. Stripping of labeled probes was performed by pouring boiling 0.1× SSC/0.1% SDS onto the blot. After slow cooling to room temperature, the stripped blots were prehybridized and hybridized as described above. A full-length human MCP-1/JE cDNA was used in Northern analysis.25

Each Northern analysis was performed at least 3 times with the exception of the analysis of neutrophils, which was performed once.

Nuclear run-on transcription assays. Nuclear run-on assays were performed as previously described.26 Slot blots were made with the followingDNAs: the plasmid pGEM-11Z(f); a full-length MCP-1 cDNA27; a 3.1-kb Sph I/Xho I fragment containing the full-length CD18 cDNA;27 a 2-kb fragment of the chicken actin cDNA29; a 1.4-kb full-length rat GAPDH cDNA29; a 1.6-kb EcoRI/Cla I fragment of the human c-myc exon 3 in pSP65;28 and a 1.3-kb EcoRI fragment containing the 3’ end of the myeloperoxidase cDNA.31 Autoradiograms were exposed at ~80°C using a Dupont lighting Plus intensifying screen and were quantitated with an LKB scanning densitometer.

Purification and culture of human peripheral blood neutrophils and monocytes. Blood was drawn by venipuncture from normal human volunteers. Neutrophils and mononuclear cells were purified by dextran sedimentation followed by Ficol-Hypaque centrifugation of the buffy coat as described.32 Neutrophils isolated from the Ficol-Hypaque pellet were greater than 95% pure after hypotonic lysis of remaining red blood cells. The remaining cells were predominantly eosinophils with less than 0.5% monocytes. Purified cells were resuspended at 105/mL in RPMI supplemented with 10% heat-inactivated FCS (Hyclone; endotoxin tested) and 100 U/mL penicillin/streptomycin ( Gibco BRL). Cells were incubated for 20 hours at 37°C. In some experiments, human granulocyte-macrophage colony-stimulating factor (GM-CSF; a gift from J.D. Griffin, Dana-Farber Cancer Institute, Boston, MA) or RA was added at concentrations of 5 ng/mL and 5 µmol/L, respectively. Monocytes were purified from the Ficol-Hypaque interface by adherence. Viability of monocytes and neutrophils, determined by the trypan blue exclusion method, was greater than 95%, both before and after incubation in medium with serum ± GM-CSF or RA.

RT-PCR analysis. Total RNA was isolated from neutrophils according to the single-step isolation method using Tri-Reagent ( Molecular Research Center, Inc, Cincinnati, OH) as recommended by the manufacturer. First strand cDNA was synthesized from approximately 1 µg of DNaseI-treated total RNA (DNaseI digested as described above for DD-PCR) using 200 U of M-MLV reverse transcriptase in a 20 µL reaction containing, 1 mmol/L dNTPs, 1 U/µL RNasin, 100 pmol/L of random hexamers (Pharmacia), and 10 mmol/L DTT in 1× buffer (50 mmol/L Tris-HCl [pH 8.3], 75 mmol/L KCl, 6 mmol/L MgCl2). The reaction was incubated at 23°C for 10 minutes, at 42°C for 60 minutes, and at 94°C for 5 minutes. PCR was performed with 2 µL of the RT product in a 30-µL reaction volume containing 40 pmol of each primer, 200 µmol/L dNTPs and 2 U of Taq polymerase in 1× buffer (50 mmol/L Tris-HCl [pH 8.3], 50 mmol/L KCl, 1.5 mmol/L MgCl2, 0.001% gelatin). Each cycle of PCR contained 1 minute of denaturation at 95°C, 1 minute of primer annealing at 48°C for the MCP-I primers (5’-CTCTGTCGCTCATAAGACGC-3’ and 5’-GTCTCAAGTCTTCCAGGATG-3’) or 1 minute at 50°C for the GAPDH primers (5’-CCATGGGAAAGCTGGGCG-3’ and 5’-CAAGATGTGTCATTGATGACC-3’), and 90 seconds of extension at 72°C. The annealing temperatures were 3°C below the Tm of each primer set. Controls for DNA contamination included a “no DNA” control in which water was used in the RT-PCR instead of cDNA; a “no RNA” control in which the RT-PCR was performed using cDNA synthesized from an RT reaction containing water instead of RNA; and a “no RT” control in which RT-PCR was performed with RNA that had not been reverse-transcribed. To insure that the RT-PCR signal obtained was linear in respect to input RNA, we performed a kinetic analysis by varying the number of cycles of amplification for each primer pair as well as by varying the amount of input RNA. Based on this empirical analysis, we found that 20 cycles of PCR using the GAPDH primers and 30 cycles using the MCP-1 primers were optimal for a linear signal. RT-PCR products were separated on 1% agarose gels and blotted to Biortran+ membrane (ICN) in 0.4 N NaOH. Blots were air-dried, prehybridized, and hybridized as described above for Northern blots using GAPDH and MCP-1 cDNAs probes.

Immunocytochemistry. Peripheral blood neutrophils and adheren-
serum (Vector Laboratories, Burlingame, CA) for 30 minutes, followed by incubation with either rabbit anti-MCP-1 (1:500) or normal rabbit serum (1:500) for 60 minutes. Subsequent incubations and washes were performed using the Vectastain antirabbit ABC kit (Vector Laboratories) according to the manufacturer’s recommendations, with the addition of 0.05% Tween-20 to the PBS wash solution. The slides were incubated with the secondary antibody for 30 minutes at 37°C and with the ABC/HRP reagent for 45 minutes at room temperature. After the final wash, monocytes and neutrophils were stained for 10 and 5 minutes, respectively, using a 1 mg/mL solution of diaminobenzidine tetrahydrochloride in a buffer containing 50 mmol/L Tris-HCl (pH 7.6), 150 mmol/L NaCl, 1 mol/L imidazole, and 0.3% NaN3. A total of 10 μL of a 30% hydrogen peroxide solution was added to 10 mL of the diaminobenzidine tetrahydrochloride solution immediately before use. The slides were counterstained with Mayer’s hematoxylin, dehydrated in ethanol, and mounted with coverslips. Immunocytochemistry was performed twice independently.

RESULTS

Identification of RA inducible genes in HL-60 cells. We have used the recently described technique of DD-PCR to identify RA-responsive genes in HL-60 cells. By comparing RNA from different time points of RA treatment, we have been able to identify a number of differentially expressed genes, as well as obtain information about the kinetics of the RA response. Using 80 different primer pairs, we identified 15 cDNAs that were differentially expressed based on DD-PCR. Three DD-PCR gels are shown in Fig 1. The reactions on these gels were judged to be evenly loaded based on the intensity of bands that appear in all lanes. However, in each gel, bands were observed that appeared to be upregulated during the course of RA treatment (indicated by arrows in Fig 1).

The 15 DD-PCR bands containing cDNAs from poten-

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sequence analysis of the 3 DD-PCR products showed that two of the cDNAs were previously uncharacterized, whereas the third was identical to the previously characterized cDNA for MCP-1. Further characterization of longer cDNAs for the 1.1-kb and 5.5-kb messages showed no similarities with sequences in the DNA and protein databases. MCP-1 is a recently described cytokine that is chemotactic for monocytes, but not for neutrophils. The MCP-1 gene is an immediate early response gene that responds to a number of stimuli in a variety of immune and nonimmune tissues.

The MCP-1 gene is transcriptionally upregulated in re-
Fig 6. Purified peripheral blood neutrophils express MCP-1 protein after incubation in serum or GM-CSF. Peripheral blood neutrophils and monocytes were isolated as described in Materials and Methods, and immunocytochemistry was performed after incubation in media alone (including serum) or medium plus GM-CSF at 37°C for 20 hours. (A) Monocytes in serum only, normal rabbit serum (control); (B) monocytes in serum only, MCP-1 antisera; (C) freshly isolated neutrophils, normal rabbit serum; (D) freshly isolated neutrophils, MCP-1 antisera; (E) neutrophils in serum plus 5 ng/mL GM-CSF, normal rabbit serum; and (F) neutrophils in serum plus 5 ng/mL GM-CSF, MCP-1 antisera.

Response to RA. Nuclear run-on analysis was performed to determine if the increase in mRNA levels was caused by transcriptional upregulation or an increase in message stability. Comparison of control HL-60 cells and 48-hour RA-treated HL-60 cells indicated that the MCP-1 gene was upregulated 20-fold when compared with the GAPDH control or 16-fold when compared to actin control (Fig 3). No significant hybridization was observed to the empty plasmid control, whereas MPO and c-myc expression were downregulated and CD18 was upregulated, as expected. The 1.1- and 5.5-kb RA-responsive genes were not transcriptionally upregulated (data not shown), suggesting that an increase in
transcript stability was responsible for the observed increase in steady-state transcript levels.

MCP-1 is upregulated in NB4 cells in response to RA. Although RA induces differentiation of HL-60 cells toward the neutrophilic lineage, it has also been shown to induce the expression of the monocyte-specific macrophage-CSF receptor. Because MCP-1 expression has been shown in monocytes, but not previously in neutrophils, it is possible that MCP-1 upregulation in RA-treated HL-60 cells represents ectopic expression of a monocyte/macrophage marker. To address this issue, we examined MCP-1 expression in NB4 cells, which also differentiate into neutrophils in response to RA. As with HL-60 cells, steady-state transcript levels for the MCP-1 gene increased after RA treatment and reached peak levels after 48 hours of treatment (Fig 4).

MCP-1 is expressed by neutrophils after culture at 37°C. To determine if normal human neutrophils express the MCP-1 gene, Northern analysis and RT-PCR were performed with total RNA from freshly isolated neutrophils and from neutrophils incubated for 20 hours at 37°C in medium containing 10% FCS ± RA. MCP-1 transcripts were undetectable in freshly isolated neutrophils when assayed by both RT-PCR (Fig 5A) and Northern blot (Fig 5B), but transcript levels increased dramatically after 20 hours at 37°C. It appears that MCP-1 transcript levels increase in neutrophils because of an activation event caused by the purification and/or culture conditions. Activation of neutrophils under such conditions has been shown by others. However, treatment with RA did not increase MCP-1 transcript levels above those of medium alone.

Immunocytochemistry was performed to eliminate the possibility that contaminating monocytes were responsible for all of the MCP-1 RNA detected in the neutrophil fraction and to directly show the production of MCP-1 protein by neutrophils. As reported previously, polyclonal antisera directed against human MCP-1 recognized a cytosolic protein in monocytes incubated at 37°C in 10% fetal bovine serum (Fig 6). In parallel with the upregulation of MCP-1 mRNA in neutrophils (Fig 5), neutrophils incubated for 20 hours, either in serum alone (data not shown) or with an established activator of neutrophils (GM-CSF), stained positively for MCP-1, whereas slides treated with normal rabbit serum remained unstained (Fig 6). MCP-1 protein could not be detected in freshly isolated neutrophils.

**DISCUSSION**

The identification of genes that are transcriptionally regulated during RA-induced neutrophil differentiation and activation is of particular importance, because the mechanism of this induction is unknown. Using DD-PCR, we have identified RA-responsive genes in the HL-60 cell line, including two previously uncharacterized genes whose products and roles have yet to be determined. Our DD-PCR screen was probably not exhaustive, because, theoretically, 300 individual primer pairs would be required to have a 95% confidence of observing all transcripts in a cell.

In the course of this screening procedure, we discovered that the MCP-1 gene is upregulated during RA treatment of human myeloid cell lines and that MCP-1 transcript and protein levels increase in normal human neutrophils when cultured in vitro. MCP-1 is a recently described cytokine that is chemotactic for monocytes but not for neutrophils. Cloning and characterization of human MCP-1 indicated that it was the human homolog of the murine JE gene, which was originally identified as a platelet-derived growth factor-inducible gene. The MCP-1 gene responds to a number of stimuli in a variety of tissues including blood mononuclear cells, endothelial cells, epithelial cells, fibroblasts, osteoblasts, and smooth muscle cells. The observation that transcripts were undetectable by Northern analysis at early time points during RA induction of HL-60 cells differs from previous reports of MCP-1 expression in other cell types, in which it is upregulated more rapidly. However, transcripts were apparent by as early as 2 hours by DD-PCR, a more sensitive assay, indicating that, although the magnitude of the upregulated expression may vary, the time course is similar.

We clearly show, for the first time, that neutrophils express MCP-1 after stimulation. The increase in MCP-1 transcripts in purified neutrophils after incubation at 37°C may be because of activation caused by the purification procedure and/or the 37°C incubation. Such a stimulatory effect has been described by other investigators while examining surface expression of CD35 (CR1) and CD11b (CR3) on purified neutrophils. The observation that RA does not appear to increase MCP-1 expression in neutrophils could be attributed to the stage of differentiation. RA may play a greater role in the regulation of MCP-1 in immature cells than it does in fully differentiated cells. We are currently examining the effect of lipopolysaccharide, FMLP, interleukin-8, and other cytokines on MCP-1 expression in neutrophils.

MCP-1 is a potent activator of monocytes and macrophages and can induce a respiratory burst in monocytes, induce the release of lysosomal enzymes, and induce monocyte-mediated inhibition of tumor cell growth. Based on these findings, it has been suggested that MCP-1 may play an important role in cellular immune reactions and response to tissue damage.

These data point to an interesting cross-talk between neutrophils and macrophages. Previously, it has been shown that activated monocytes can produce interleukin-8 to attract and activate neutrophils. Here, we show that a reciprocal relationship exists in which neutrophils can produce MCP-1 to attract and activate monocytes. These data fit nicely into the current concept of inflammation, in which granulocytes are the first line of defense and are present in nearly all forms of inflammation, whereas monocytes are responsible for the control and continuation of the immune reaction. Therefore, MCP-1 expression by stimulated neutrophils may play an important role in host defense and repair of tissue damage by recruiting monocytes to the site of inflammation.

**ACKNOWLEDGMENT**

We would like to thank Michel Lanotte for the NB4 cell line; Linda Clayton for oligonucleotides used in DD-PCR; David Gonzalez for synthesis of oligonucleotides; Maria Teresa Voso for her assistance with the neutrophil purification and RT-PCR; Steven Ackerman and Kathy Earnst for assistance with the immunostaining;
and Dong-Er Zhang, David Mastrianni, Maria Teresa Voso, and Jozef Jansen for their suggestions during the course of this work.

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